PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Taylor *et al.*	Group Art Unit: 1638
Serial No.: 09/386,605	Examiner: Page, Brent T.
Filed: August 31, 1999	Atty. Dkt. No.: 11000023-2230 MONS:131US
FOR: NOVEL TRANSGENE ASSAY USING STABLE *AGROBACTERIUM RHIZOGENES* TRANSFORMATION	

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March 17, 2008 — Date
/Robert E. Hanson/ — Robert E. Hanson

BRIEF ON APPEAL

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BRIEF ON APPEAL

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief. The date for filing this Brief is March 17, 2008. The fee for filing this Brief is being concurrently filed. Should any additional fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, or should an overpayment be made, the Commissioner is authorized to deduct or credit said fees from or to Sonnenschein Nath & Rosenthal LLP Deposit Account No. 19-3140/MONS:131US.

I. REAL PARTY IN INTEREST

The real party in interest is Monsanto Company, the parent company of assignee Monsanto Technology LLC.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-26 were filed with the application. Claims 2-7 were canceled and claims 12-26 are withdrawn as being directed to a non-elected restriction group. Claims 1 and 8-11 are therefore currently pending and under examination. Claims 1 and 8-11 were rejected by the Examiner in the Final Action dated October 18, 2007 and are the subject of this appeal. A copy of the appealed claims as they currently stand is provided in Section VIII.

IV. STATUS OF AMENDMENTS

An amendment to claims 1, 8 and 11 was made in the Response to Office Action filed on June 11, 2002 and was entered by the Examiner. An amendment to claim 1 was made in the Response to Office Action filed on December 2, 2003 and was entered by the Examiner. An amendment to claims 1 and 8 was made in the Response to Office Action filed on April 11, 2005 and was entered by the Examiner. An amendment to claim 1 was made in the Response to Office Action filed on April 20, 2006 and was entered by the Examiner. An amendment to claims 1 and 8 was made in the Response to Office Action filed on November 20, 2006 and was entered by the Examiner. An amendment to claim 1 was made in the Response to Office Action filed on July 27, 2007 and was entered by the Examiner. No subsequent amendments have been filed.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 1 relates to a method for producing a stably transformed chimeric dicotyledonous plant having transgenic root tissue. Specification from page 3, line 10 to page 3, line 12. The method comprises the steps of:

obtaining a stem or hypocotyl explant from a selected dicotyledonous plant species, wherein the hypocotyl explant has a cut end below the cotyledon (Specification from page 7, line 15 to page 7, line 17);

transforming the stem or hypocotyl explant with *Agrobacterium rhizogenes* containing an exogenous nucleic acid sequence capable of being transferred to the explant, wherein the cut end of the hypocotyl explant is contacted with the *Agrobacterium rhizogenes* (Specification from page 7, line 19 to page 7, line 21);

culturing the transformed explant in a root initiating media to produce transformed roots (Specification from page 7, line 27 to page 7, line 28); and

transferring the transformed roots to soil or a hydroponic environment to produce a chimeric dicotyledonous plant having transformed roots and wild type shoots, stems and leaves (Specification from page 7, line 31 to page 7, line 34), wherein the dicotyledonous plant is soybean (Specification from page 8, line 20 to page 10, line 31).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Were claims 1 and 8-11 properly rejected under 35 U.S.C. 103(a) as being unpatentable over Trulson *et al.* (EP 0262972 A2) in view of Simpson *et al.* (1986 *Plant Mol. Biol.* 6:403-415) and further in view of Savka *et al.* (1990 *Phytopathology* 80:503-508)?

VII. ARGUMENT

A. The Claims Are Not Obvious Because the Combination of Trulson *et al.*, Simpson *et al.* and Savka *et al.* Do Not Teach or Suggest All Claim Elements

The Examiner asserts that Trulson *et al.* teaches a method of producing a stable transformed chimeric cucumber plant having transgenic root tissue, by obtaining a hypocotyl explant, inoculating the explant with *Agrobacterium rhizogenes* and producing plants. Trulson *et al.* is further asserted to demonstrate chimeric cucumber plants at page 6, line 45-55, describing 690 roots from *A. rhizogenes*-inoculated hypocotyl sections, where 64 roots regenerated plants, 22 of which were positive for NPTII, the transgenic selection marker. Of 11 plants that had not been selected for NPTII expression, two were said to be positive for NPTII, whereas the remaining 9 did not have the selectable marker. These are asserted to represent chimeric plants with transgenic roots and non-transgenic shoots.

The Examiner further asserts that Simpson *et al.* teaches the *A. rhizogenes* transformation of soybeans to produce transformed roots and Savka *et al.* teaches the effectiveness of *A. rhizogenes* K599 in inducing hairy roots in soybean.

However, the assertions of the Examiner are incorrect because Trulson *et al.* does not teach the production of a stably transformed chimeric plant having ***transformed roots and wild type shoots, stems and leaves***. Further, neither Simpson *et al.* nor Savka *et al.* teach transformed chimeric plants. Since the combination of references, and the art generally, do not teach or suggest each element of the claims, nor provide motivation to practice the claimed methods, the rejection under 35 U.S.C. 103(a) should be reversed.

1. Trulson *et al.* Does Not Teach The Formation of a Chimeric Dicotyledonous Plant Having Transformed Roots and Wild Type Shoots, Stems and Leaves

Trulson *et al.* recites inoculating cucumber hypocotyl sections with *A. rhizogenes* then, after one week, transferring the inoculated sections into media without kanamycin (Page 5, lines

52-62). Kanamycin is used to select for tissue that has the NPT gene, which is encoded on an Ri plasmid that is in the *A. rhizogenes* inoculum. In a successful transformation, the NPT gene is transferred to the plant where it deactivates the selective agent kanamycin.

The roots produced on the inoculated surfaces of Trulson *et al.* were excised and divided into two groups. In the first group (Series A), the excised roots were placed on media without kanamycin. The second group (Series B) consisted of roots that were placed on media with kanamycin (p. 6, lines 4-9). After 2-3 weeks, embryoids that appeared on the root surface were detached and transferred to media without kanamycin for 10-14 days to develop mature embryoids (p. 6, lines 9-11). The mature embryoids were transferred to a media without kanamycin where plantlets (with shoots) were produced. The regenerated plantlets were transplanted into soil in pots for hardening and development into plants (p. 6, lines 12-15).

Transformation of the regenerated plants was assessed by assaying leaf tissue for NPT on the plantlets before transferring the plantlets to pots, and 2-4 weeks after the transfer. FIG. 2 of Trulson *et al.* shows the results of an NPT assay on leaf tissue of 9 plants, where plants 1 and 6 had leaf tissue positive for NPT (p. 6, lines 18-26).

As discussed on page 6, line 44 - page 7, line 29 of the reference, 691 roots were harvested from the Series A and Series B groups, of which 64 regenerated into plantlets. Trulson *et al.* note that this is similar to the amount of regenerated plantlets expected from non-transformed root explants. Of those 64 plants, 22 were NPT positive in the leaf assay. In Series A (no initial kanamycin selection), 11 plants were regenerated, of which two were NPT positive in the leaf assay. Although the Examiner asserts that the nine negative Series A plants are chimeras, Trulson *et al.* did not test the roots for NPT or any other indicia of transformation. Since those plants were ***never under selection***, there is no basis to suggest that these plants were

transgenic at all. Specifically, since non-transformed root explants exhibit a similar amount of regeneration as transformed root explants, there is no reason to believe that the nine plants asserted by the Examiner to be chimeras have any transformed tissue at all. Similarly, with the Series B plants (rootlets subject to kanamycin selection), of 565 roots cultured, 53 regenerated into plantlets, of which 22 had NPT-positive leaf tissue. However, there is no basis to believe that the 31 plantlets that did not have NPT in their leaves were transformed at all. Indeed, Trulson *et al.* express a belief that these plants are not transformed, at page 7, lines 25-27, by stating, "[t]he addition of 25 mg/l kanamycin did not affect the regeneration process of the transformed tissue, ***nor did it prevent regeneration of some NPT-negative plants***." (emphasis added). It is also well known in the art that a selection regime yields some non-transgenic plants. Further, transient, non-stable transgenic expression can occur where a transgene does not stably insert into the genome. Again, Trulson *et al.* did not test the roots for NPT so there is no evidence that any of the plants were chimeras: Trulson *et al.* simply do not make any such suggestion.

In contrast to Trulson *et al.*, Applicants' method for producing a chimeric plant involves transforming a suitable explant that is capable of maintaining a non-transgenic stem, leaves and other plant structures after inoculation with *A. rhizogenes*. Specification at p. 7 ll. 12-14. An incision or wound is made in the explant, which is preferably a stem, hypocotyl or similar structure. Next, the explant is inoculated with *A. rhizogenes* at the wound site. Transgenic roots can then be induced at the inoculated end of the explant. *Id.* at p.7 ll. 15, 19-21, and 26-29. Using Applicants' claimed method, once roots begin to grow on the explant, the entire chimeric plant may be grown in soil or hydroponics rather than media because the wild type shoots, stems and leaves support the transgenic roots. *Id.* at p.7 ll. 31-34. Trulson *et al.* simply does not teach

or suggest such a method or the plants produced thereby in which chimeras having transformed roots and wild type shoots, stems and leaves are obtained.

2. Simpson *et al.* and Savka *et al.* Do Not Teach or Suggest Claim Elements Lacking in Trulson *et al.*

To establish a *prima facie* case of obviousness, the prior art references or art generally must teach or suggest all the claim limitations. M.P.E.P § 706.02(j).

The Examiner cites Simpson *et al.* and Savka *et al.* as teaching soybean plant systems which may be transformed using *A. rhizogenes*. However, because Trulson *et al.* do not teach a stably transformed chimeric dicotyledonous plant with transformed roots and wild type shoots, stems and leaves, as thoroughly explained above, the claims can only be obvious if Simpson *et al.* and Savka *et al.* teach or suggest this missing element of Applicants' invention. As explained below, this is not the case.

Savka *et al.* relate to use of hairy root cultures for propagation of soybean cyst nematodes, and the transformed tissues are maintained as root cultures. See, e.g. page 504, right column; page 507, right column. Savka *et al.* also explain that no opine-positive transformed roots were induced from hypocotyl inoculation; transformed roots were obtained from globular callus that developed at the inoculation site on cotyledons. Abstract. Thus, Savka *et al.* do not describe a successful method of obtaining transformed roots from soybean hypocotyl explants. Further, no chimeric plants are described or apparently contemplated in Savka *et al.*

Simpson *et al.* likewise describes development of transformed root clones. See, *e.g.* page 409, section entitled "Plant Transformation", including Table 2. Specifically, Simpson *et al.* state that "...we inoculated inverted stems or hypocotyls of...soybean. The resulting roots were excised and transferred to hormone-free media and grown as separate root clones." p. 409, left column, bottom paragraph, emphasis added. Additionally, Simpson *et al.* note difficulties with

soybean, particularly, "a high background of non-transformed roots." Abstract. Simpson *et al.* do suggest fully transformed plants might be regenerated from transformed roots. p. 411, right column, paragraph 2. Yet, nowhere do Simpson *et al.* describe production of a stably transformed chimeric plant as presently claimed.

In light of the above, Applicants respectfully submit that no combination of the cited references teach or suggest the production of a stably transformed chimeric dicotyledonous plant having transgenic root tissue and wild type shoots, stems and leaves. Therefore Applicants' invention is not rendered obvious from them and reversal of the rejection is therefore respectfully requested.

B. Conclusion

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

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VIII. CLAIMS APPENDIX

APPEALED CLAIMS:

1. A method for producing a stably transformed chimeric dicotyledonous plant having transgenic root tissue, the method comprising the steps of:

obtaining a stem or hypocotyl explant from a selected dicotyledonous plant species, wherein the hypocotyl explant has a cut end below the cotyledon;

transforming the stem or hypocotyl explant with *Agrobacterium rhizogenes* containing an exogenous nucleic acid sequence capable of being transferred to the explant, wherein the cut end of the hypocotyl explant is contacted with the *Agrobacterium rhizogenes*;

culturing the transformed explant in a root initiating media to produce transformed roots; and

transferring the transformed roots to soil or a hydroponic environment to produce a chimeric dicotyledonous plant having transformed roots and wild type shoots, stems and leaves, wherein the dicotyledonous plant is soybean.

8. The method of claim 1 wherein transformed roots are initiated in the hypocotyl by placing the end of the hypocotyl contacted with the *Agrobacterium rhizogenes* in a media containing ¼ strength Murashige and Skoog media.

9. The method of claim 8 wherein the media further comprises a selectable agent.

10. The method of claim 9 wherein the selectable agent is kanamycin.

11. The method of claim 10 wherein the concentration of kanamycin in the media is no more than 50 mg/L.

IX. EVIDENCE APPENDIX

Exhibit A: Trulson *et al.*, "Genetic transformation and controlled regeneration of cucumis SP in vitro." European Patent Publication No. 0262972, published June 4, 1988. Cited by Examiner.

Exhibit B: Simpson *et al.*, "A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes*," *Plant Molecular Biology* 6: 403-415, 1986. Cited by Examiner.

Exhibit C: Savka *et al.*, "Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode," *Phytopathology* 80: 503-508, 1990. Cited by Examiner.

EXHIBIT A

09/386,605

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(54) Genetic transformation and controlled regeneration of cucumis SP in vitro.

(57) Transformation of Cucumis sp. plants with A. rhizogenes followed by regeneration into genically altered plants. Inverted hypocotyls of C. sativus L., cv. Straight Eight were inoculated with A. rhizogenes containing the vector pARC8 or pARC16 containing NOS/NPT chimeric marker gene conferring resistance to kanamycin in addition to the resident Ri plasmid. Roots produced as the inoculated hypocotyls were excised and sequentially regenerated in CTM-2, -3 and -4 media, resulting in mature, fruiting transgenic plants having germinable seed. Short intermodal morphology was expressed yielding Cucumber plants, called "PCRI HI Density", having greater yield per acre.

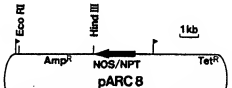

Fig. I.

EP 0 262 972 A2

Bundesdruckerei Berlin

Description

"GENETIC TRANSFORMATION AND CONTROLLED REGENERATION OF CUCUMIS SP IN VITRO"

Cross Reference to Related applications:

The genetic transformation methods of this invention may be followed by explant regeneration techniques set forth in our copending explant regeneration case (our reference N 43003, hereinafter referred to as case 92/9) filed of even date herewith, or by protoplast isolation and regeneration techniques set forth in our copending protoplast regeneration case (our reference N 43004, hereinafter referred to as case 92/13) filed of even date herewith. Likewise, the somaclonal variants produced by the techniques of case 92/9 or somatic hybrids produced by protoplest/cytoplast/sphereoplsast/liposome fusion (92/13) may be preceded by and combined with the transformation techniques of this case. The disclosures of those cases are hereby incorporated by reference herein to the extent needed.

Field:

This invention relates to genetic transformation of Cucumis sp. plants by use of Agrobacterium rhizogenes and subsequent regeneration in vitro into genetically altered plants. More specifically, this invention relates to transformation of Cucumis sp. plants by inoculation of inverted hypocotyl sections with A. rhizogenes, induction of roots thereon, and regeneration of genetically altered plants from the root tissue. The regeneration can be achieved directly from the induced roots, or from explents of tissue from the roots, stem, or leaves of plantlets or meture plants, employing CTM-1 to -3 culture media, or from protoplests isolated from such tissue employing CMP-1 to -3 media. The transformation techniques of this invention, when combined with regeneration, somatic hybridization, and somaclonel veriation screening, permit a complete range of techniques for genetic improvement in plants, and more specifically, in the genus Cucumis. Transgenic plants of this invention called "PCRI HIDensity" are characterized as having a compact morphology (short internodes).

Background

Sterility barriers between species are among the most limiting factors in plant breeding. They preclude transfer of many desirable traits such as disease, insect and herbicide resistance between species of cultivated or week plants because of sexual incompatibility. This problem is especially acute in the family Cucurbitaceae, which includes the genera Cucumis (cucumbers [C. sativus], and melons [C. melo]), Citrullus (watermelons) end Cucurbita (squesh.) Among the cultivated species of the genus Cucumis, successful sexual crosses can be mede only between C. setivus and the closely releted C. herwickii. Attempts to interbreed cucumbers and other species in this genus failed (Deakin et el., 1971).

The recent advances in genetic engineering seem to be a promising alternative to sexual propagation techniques for improvement of this economically important crop. This is particularly true in the case of tissue culturing where en increase in genetic variation, so-called somaclonal variation, Is noted (Larkin and Scowcroft 1981). Further, sterility barriers may be overcome by fusion of protoplasts of sexually incompatible species. However, the lack of a reliable method for regeneration of Cucumis plants by explent tissue culture techniques has prevented significant progress until our recent developments of both: a) the CTM-1 through CTM-4 series of explant embryoid generation and development media, as reported by us in Trulson, A.J. and Shahin, E.A., "In vitro plant regeneration in the genus Cucumis" (in press 1986) and covered in our copending application (92/9); and b) the CPM-1 through CPM-3 series of protoplast regeneration and development media, also reported by us in the aforesaid paper (Trulson and Shahin, in press 1986) and covered in our copending application (92/13).

Still another route to overcoming sterility barriers is the introduction of desirable traits by gene transfer and subsequent expression thereof. Inter- and intraspecific gene transfer is a major objective of genetic engineering. Successful expression of transferred genes in transformed plants is possible and has already been achieved in several systems (Fraley et al., 1983; Herrera-Estrella et al., 1983; Bevan 1984; Horsch et al., 1985; Jones et al., 1985). This success is the basis for the great hopes for future crop improvement through the incorporation of various desirable traits. The application of genetic engineering would be especially welcome in the improvement of the genus Cucumis, particularly cucumbers, since sexual incompatibility between cucumbers and other members of the family Cucurbitaceae precludes transfer of such traits as disease and insect resistance using convention breeding methodology (Deekin et al., 1971).

The most widely used method of gene transfer is via a disarmed form of the Ti plasmid of the soil bacteria Agrobacterium tumefaciens (Zambryski et al., 1983). A. tumefeciens is a plant pathogen that causes crown-gall tumors after infection of wounded dicotyledonous plants. Large plasmids (Ti-plasmids) are responsible for the oncogenicity of the bacterium. Crown-gall tumours contain a DNA segment, called the T-DNA, which is homologous with a defined part of the Ti-plasmid present in the tumor-inducing bacterium, and is stably integrated into the plant genome. Apart from the T-DNA, another region of the Ti-plasmid-called the vir-region, is essential for tumour induction (Hoekema et al., 1983). An alternative tool for gene transfer is A. rhizogenes, which differs from A. tumefeciens by inducing roots rather than tumours (Chilten et al. 1982; David et al., 1984).

Binary Ti plasmid vector systems consist of two plasmids in Agrobecterium, where one plasmid contains the DNA that can be transferred to plant cells and the other contains the virulence (vir) genes which are necessary for the DNA transfer but are not themselves transferred. Hoekema et al. (1983) reported the interaction in A. tumefeciens of two compatible plasmids, one containing the vir-region, the other carrying the T-DNA on a wide host-range replicon. An A. tumefaciens strein harboring both plasmids has a normal tumor-inducing capacity, although neither plasmid is functional alone. With this approach, the T-DNA on one plasmid can, because of it size, be easily genetically menipuleted using Escherichia coll as e host. Transfer of this plesmid into an A. tumefeciens strain harboring the plasmid with the vir-region allows introduction of the manipuleted T-DNA into plent cells. In this wey, sophisticated binery vector systems for plant genetic engineering can be developed.

Simpson et al. (in press 1986) described two non-oncogenic vectors (pARC4 and pARC8) based on the binary Ti plesmid system of Agrobecterium tumefeciens for plant transformetion. Each vector contains the left and right termini sequences from pTiT37. These sequences, which determine the extent of DNA transferred to plent cells, flank unique restriction enzyme sites and a merker gene that functions in the plant (nopaline synthase in pARC4, or neomycin phosphotransferase in pARC8). After construction in vitro, the vectors can be conjugetively transferred from E. coli to eny of several Agrobecterium strains containing vir genes.

Using A. rhizogenes strain A4 containing the resident Ri plasmid plus a vector with the nopaline synthase marker, Simpson et al (in press, 1986) found that up to 50% of the hairy roots resulting from the infection of alfalfa or tomato synthesized nopaline Vector DNA encoding a screenable marker was frequently co-transferred with Ri plasmid DNA to an alfelfe or a tomato cell. In contrast, they found the frequency of co-transfer to soybean cells difficult to estimete beceuse they encountered e high beckground of non-transformed roots using that species. Up to five copies of the vector DNA between the terminal sequences were faithfully transferred and maintelned in most cases suggesting thet the termini sequences and the vir genes from the Ri and Ti plasmids are functionally equivelent. Simpson et al (in press 1986) did not study the femily Cucurbiteceae or the genus Cucumis.

To our knowledge no one has been able to successfully transfer genetic meterial into Cucumis sp. plent tissue via either A. tumefeciens or A. rhizogenes with regeneretion into e genetically alterad plant structure (cell, cell colony, tissue, mini-calli, embryoid, plantlet, or plent) which expressed tha transferred gene. Thus, to dete, this avenue of genetic engineering for Cucumis sp. has remained closed.

Accordingly, there is a need for an efficient, facile and reproducible method by which A. rhizogenes can be used successfully to transfer a foreign gene into Cucumis sp. plant structures or plants, which methodology opens new avenues in genetic improvement of Cucumis sp. by permitting the transfer of agronomically desirable genes. Additionally, there is a need for a technique for introduction of marker genes, such as kanamycin resistance, to facilitate somatic inter- and intraspecies hybridization/cybridization of Cucumis sp. with other species.

THE INVENTION

Objects

It is among the objects of the invention to provide methods for the genetic transformation of plant structures and/or plents by use of Agrobecterium rhizogenes, and more particulerly, transformetion of Cucumis sp. plant tissue.

It is another object of the invention to provide methods of in vitro regeneration of transformed Cucumis sp. plant tissue into competent, genetically altered mini-calli, embryoids, roots, plentlets and plents.

It is enother object of this invention to provide processes for the in vitro generetion, induction and regeneration of cells, cell colonies, mini-calli, embryoids and plantlets from genetically altered protoplasts or explant tissue which are capeble of developing into mature plants.

It is another object of this invention to provide methods of continuously producing genetically altered plant mini-calli and embryoids for encapsulation as artificial seeds.

It is another object of this invention to epply the novel methods and media of this invention to the production of improved plants in the genus Cucumis.

It is another object of this invention to shorten the time for producing genetically engineered and/or hybridized plant stock without having to go through full seed development.

It is another object of this invention to asaxuelly propagata plents without hormonal or chemicel induction of flowers.

It is another object of this invention to provide methods for introducing foreign or selectable merker genes into Cucumis sp., inter alia, to facilitate somatic hybridization.

It is another object of the invention to produce geneticelly altered mini-calli end embryoids which mey be stored frozen, followed by later thawing and continuetion of maturation and development into genetically altered plentlets and/or plants.

It is another object of this invention to provide methods of transforming Cucumis sp. tissue to facilitate esexuel sometic hybridization.

It is another object of this invention to introduce one or more vector T-DNA(s) alone, the Ri-plasmid T-DNA alone, or both vector and Ri plasmid T-DNA(s), of A. rhizogenes into Cucumis sp. plants with expression of one or the other, or both.

It is another object to introduce resistance to kanamycin, chloramphenicol, or other antibiotics, to Cucumis

sp. plants as marker genes for genetic engineering and trait selection.

Still other objects of this invention will be evident from the balance of this specification and claims.

Definitions

The terminology used herein is not intended to vary from the terminology used in the field. However, the meaning of some terms used in the field is not necessarily uniform, and the following definitions will be of help in this case:

"Transformation refers to genetic alteration of a plant by introduction, and stable and heritable incorporation, into the subject plant DNA of a foreign (plant, bacterial, viral or chimeric) DNA.

"Plantlet refers to a plant sufficiently developed to have a shoot and a root that is asexually reproduced by cell culture.

"Explant refers to a section or a piece of tissue from any part of a plant for culturing.

"Hormone" refers to a plant growth regulator that affects the growth or differentiation of plants, and is exogenous as used in reference to the various media herein.

"Callus", and its plural "calli" refer to an unorganized group of cells formed in response to a cut, severing or injury of a plant, and herein refers to the unorganized cell growth which may form on explant tissues during culturing or from division of protoplasts which have regenerated cell walls.

"Embryoid refers to a structure similar in appearance to plant zygotic embryo.

"Somatic Hydrid" and "Somatic Hybridization" refers generally to stable combination of cellular material, be it protoplast/protoplast or protoplast/cytoplast combinations, and includes cybrids and cybridization.

Abbreviations

NAA = alpha-naphthaleneacetic acid
ZR = Zeatin Riboside
BAP = 6-benzylaminopurine
GA_3 = Giberellic acid
2,4-D = 2,4-dichlorophenoxyacetic acid MS Medium - Murashige and Skoog Medium (Murashige and Skoog, 1962)
CPM = Cucumis Protoplast Medium, as in CPM-1, -2, -3 (Trulson and Shahin, 1986)
CPE = Cucumis Protoplast Enzyme solution
CPE-G = Cucumis Protoplast Enzyme solution containing glycine
CTM = Cucumis Transformation Medium, as in CTM-1, -2, -3, -4 (Trulson and Shahin, 1986)
PET Solution = Tomato pre-enzymatic solution (Shahin, 1985)
TM-1 = Tomato Medium 1 (Shahin, 1985)
TM-2 = Tomato Medium 2 (Shahin, 1985)
pARC8 = A binary vector introduced in A. rhizogenes (Simpson et al., 1986)
pARC16 = A modified pARC8 binery vector having a 9.0 kb Hind III fragment at the Hind III site of the T-DNA
Hind III = A well-known restriction enzyme
AB Medium = Agrobacterium Medium (Chilton et al., 1974)

Drawings

The disclosures herein have reference to the drawings in which:

Figure 1 is a schematic of the pARC8 vector plasmid used in the transformation of this invention;

Figure 2 is a reproduction of the NPT II test to select for kanamycin resistance (+) or absence of resistance (-);

Figure 3 is a reproduction of the Southern blot Hind III fragment analysis demonstrating integration of the Vector DNA into the plant genome; and

Figure 4 is a photographic reproduction of a transgenic plant of this invention compared to a normal control.

Summary

Transgenic cucumber plants were regenerated from roots induced by inoculation of inverted hypocotyl sections of Cucumis sativus L., cv. Streight Eight with Agrobacterium rhizogenes containing the vector pARC8 or pARC16 in addition to the resident RI-plasmid.

Roots produced on the surfaces of inverted hypocotyls inoculated with A. rhizogenes were excised, and a first series were placed on CTM-2 medium (comprising MS salts, 5µM 2,4-D 5 µ M NAA and 2 µM BAP in 0.7% agar), followed by culturing for 2 - 3 weeks under continuous light (3500 lux), at 27°C. A second series of inoculated roots were cultured on CTM-2 medium supplemented with 25 mg/l of kanamycin (Sigma). Embryoids that appeared on the root surface were detached and transferred to CTM-3 Medium (MS with 5µM NAA and 2µM BAP in 0.7% agar), and cultured in the same conditions for 10 - 14 days. Mature embryoids were transferred onto hormone-free MS medium (1% agar) on which shoots were produced. To eliminate bacterial carry-over, media contained 100 mg/l of cefotaxime.

The DNA transferred to the plant from the vector (T-DNA) included a gene which encoded the enzyme neomycine phosphotransferase II, and thus conferred on the plant cells resistance to kanamycin. The

transgenic plants looked normal and were positive for the neomycin phosphotransferase II. Southern blot analysis of the transgenic plants revealed that all plants contained vector DNA, but only some of them contained DNA from the Ri plasmid.

The transgenic plents of this invention produced fruit having germinable seed which has been carried into the R-2 generation, and is called "PCRI HiDensity", plants of which are characterized as having a compact morphology (short internodes) permitting closer plant spacing, with attendant greeter production per acre.

Figure 1 shows a schematic of the disarmed pARC8 vector plasmid used in the transformations of this invention. The vector contains a wide host renge replicon (to permit the replication of the plasmid in both Escherichia coli and Agrobacterium), a bacterial origin of transfer (which permits the vector to be mobilized by a helper plasmid), and markers encoding resistance to tetracycline (TET^R) and ampicillin (Amp^R), which allow selection of bacteria containing the vector. The unique restriction enzyme sites Eco RI and Hind III facilitate the insertion in vitro of "foreign DNA fragments into the vector. The flegs merk the terminl sequences which Agrobacterium uses to delimit the DNA it transfers to plants cells. The NOS/NPT is a selectable marker which confers resistance to kanemycin in transformed plant cells. This chimeric gene consists of the neomycin phosphotransferase (NPT) coding region, flanked by the nopaline synthase (NOS) promoter and terminator. The pARC16 is a modified pARC8 vector.

Detailed Description of the Best Mode of Carrying Out the Invention

The following detailed description is by way of example and not by way of limitation of the principles of this invention, and has reference to specific examples in which transformation by A. rhizogenes followed by regeneration, embryoid generation from induced roots and plantlet development therefrom, within the scope of this invention is described for cucumber (Cucumis sativus L.) by way of example.

EXAMPLE A. Transformation of Cucumis sp.

1. Medie Composition

The composition of the various media employed herein are as set forth in the corresponding references given above, unless otherwise noted herein.

2. Source of Cucumis Hypocotyl Sections for Inoculation.

Seeds of cucumber cv. Straight Eight (ARCO Seed, Brooks, Oregon) are sterilized in 10% (v/v) Clorox (commercial bleach conteining 5.25% sodium hypochlorite) with a drop of Tween 80 (one droplet per 100 ml of the sterilizing solution) for 10 minutes, then rinse 3x in sterile, distilled water and placed in sterile petri plates (ca 30 per plete) lined with moist Whatman #3 filter peper. To assure uniform end rapid germination of the seeds, plates are kept for 24 - 30 hours at 27°C, in the dark. Germinated seeds (radicle length ca 5mm) ere pleced aseptically in Magenta boxes (six seeds per box) containing ca 40 ml of TM-1 medium (Shahin, 1985) supplemented with 150 mg/l of Carbenicillin (Sigma), and incubated for four days in a growth chamber, at 21°C night, 28°C day, 14 hour photoperiod (4500 lux). When the seedlings are green, but the cotyledons only partlely unfolded, Magenta boxes are placed for 14 hours in the dark, at room temperature.

3. Vector plasmid end Agrobacterium strain.

We used the A4 strain of A. rhizogenes conteining, in eddition to the resident Ri-plasmid, a vector pARC8 (see Figure 1) derived from the Ti plasmid of A. tumefeciens (Simpson et al., in press 1986), or e vector pARC16, which is a modified pARC8 vector in which a 9.0kb Hind III restriction fragment inserted at the Hind III site of the T-DNA region. The methods and binery vector of this system is disclosed and claimed in copending application Serial Number 634,283, filed July 25, 1984, the disclosure of which is hereby incorporated by reference herein to the extent required. As shown in Figure 1, the selectable marker in pARC8 (and in pARC16), which conferred resistance to kanamycin, is NOS/NPT, a chimeric gene constructed from Tn5 neomycin phosphotransferase (NPT) coding region flanked by the nopeline synthase (NOS) promoter end terminator.

4. Inoculation Procedure.

The inoculum consisted of four-day-old culture of the above A-4 (pARC8 or pARC16) A. rhizogenes strain, grown et room tempereture in the derk, on AB medium (Chilton et al., 1974) supplemented with 5 mg/l of tetracycline (Sigme) to select for the becteriel Tet^R merker on the vector. The inoculum was collected on sterile becteriological loop and smeered gently on the cut surface of inverted hypocotyl sections (approx. 2 cm long) of the seedlings of 1 above which were placed in hormone-free MS medium (Murashige and Skoog, 1962). The plates were then sealed and incubated at 27°C, under continuous light (2500 lux). One week after inoculation, the hypocotyl sections were cut above the ager surfece and transferred into hormone-free MS medium supplemented with 100 mg/l of the antibiotic cefotoxime (Calbiochem), followed by incubation in the seme conditions. Cefotaxime, an ampicillin anelogue, was used since it is not modified by the beta-lectamese encoded by the Amp^R gene on the vectors pARC8 or pARC16.

5. Plant regeneration using CTM Media. Test Series A and B.

In all media below, the pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 minutes. To eliminate bacterial carry-over, the media contained 100mg/l of cefotaxime.

In a first series of tests (Series A), roots (5 to 10 mm in length) produced onthe inoculated surfaces were excised and placed on CTM-2 Medium (comprising of MS salts, 5µ M 2,4-D (Sigma), 5 µ M NAA (Sigma), 2 µ M BAP (Sigma) in 0.7% agar) followed by culturing fo 2 - 3 weeks under continuous light (3500 lux) , at 27°C. In a second series (Series B), the roots were excised and cultured in CTM-2 Medium supplemented with 25 mg/l of kanamycin (Sigma) to select tranformed plants.

In both series of tests, embryoids that appeared on the root surface after 2 - 3 weeks were detached and transferred to CTM-3 Medium (MS medium having 5 µM NAA and 2µM BAP in 0.7% agar) and cultured under the same conditions for 10 - 14 days to develop mature embyroids.

These mature embryoids were transferred onto CTM-4 medium (hormone free MS medium solidified with 1% agar) on which shoots (plantlets) were produced.

These regenerated plantlets were transplanted to a mixture (1:1,v/v) of peatlite (Jiffy Products Co., West Chicago IL.) and soil for hardening and development into plants.

6. Neomycin phosphotransferase (NPT) assay.

The frequency of transformation by vector DNA was assessed in the regenerated plants of both Series A and B using an assay for NPT (Reiss et al., 1984). Small pieces (approximately 25 mm²) of leaf tissue were used in the assay. The assay was performed twice, initially on plantlets on the hormone-free CTM-4 medium, and later on plants, 2 - 4 weeks after they had been potted and hardened in the soil mixture.

Figure 2 shows the neomycin phosphotransferase II test on cucumber plants regenerated from roots induced by inoculation with A. rhizogenes in accord with this invention. We used the native polyacrylamide gel assay of Reiss et al. (1984). Numbers 1 - 9 represent samples from randomly chosen cucumber plants regenerated without selection on kanamycin, sample No. 10 is a positive control (bacteria producing NPT). "NPT" indicates mobility of the enzyme.

7. Isolation of DNA and Southern blot analysis.

Southern blot analysis (Southern, 1975) was used to confirm the integration of the NOS/NPT gene in the DNA of the NPT-positive plants. The DNA was isolated from 2 grams (fresh weight) of young leaf tissue according to the procedure of Saghai-Maroof et al. (1984), then digested with Hind III and electrophoresed on an agarose gel, blotted and probed essentially as described by Thomashow et al. (1980). Plasmid pNEO 105 (Simpson et al., 1986; copending application Serial No. 634,283) containing the chimeric gene NOS/NPT, was used as the probe of the transferred portion of the vector DNA. Southern blot analysis was also used to investigate the extent of the RI-plasmid DNA transfer into the DNA of the NPT-positive plants. Plasmids pFW94 and pFW41 (Huffman et al., 1984), which are clones of the RI plasmid T-DNA, were used as probes to determine the presence of the T_L-DNA and T_R-DNA, respectively.

Figure 3 shows the southern blot hybridization analysis of cucumber plant DNA demonstrating integration of the vector DNA into the cucumber genome. Lane 1: half-copy reconstruction of the chimeric NOS/NPT gene containing Eco RI- and Hind III- digested pNEO 105 which contains the NOS/NPT gene cloned in pBR322 (Simpson et al., 1986). Lane 2: Hind III-digested DNA from non-transformed, control cucumber plant. Lanes 3 - 6: Hind III-digested DNA from NPT-positive plants regenerated from transformed roots. The probe is pNE0105.

9. Results and Discussion

The A4 strain of A. rhizogenes containing vectors pARC8 or pARC16 infected Cucumis sp. cells, as exemplified by the cucumber hypocotyls above, as indicated by dense, cream-colored callus that appeared on inoculated surfaces after 7 - 10 days. One or two weeks later this callus produced roots. Control, uninoculated hypocotyl fragments produced small amounts of white, loose callus but did not produce roots.

A total of 691 roots harvested from the inoculated hypocotyl sections were plated on the embryo-induced CTM-2 Medium. Of these, over 9.2% (64 roots) regenerated into plantlets. This is a similar ratio as was obtained with non-transformed root explants. Among each of the 64 plantlets regenerated from a separated root, 22 plantlets were positive in the test for the neomycin phosphotransferase II (NPT-positive). These plants remained NPT-positive when assayed a second time after they were potted in the soil mix. Table I below summarizes the results.

TABLE I

Transformation of cucumber (Cucumis sativus L., cv. Straight Eight) by A. rhizogenes with and without selection on kanamycin.

Selection Agent	Number of roots cultured	Number of roots that regenerated into plantlets	Number of NPT-positive plantlets
- kanamycin	126	11	2
+ kanamycin	565	53	20
Totals	691	64	22

As noted above in Table I, without selection for resistance to kanamycin two out of eleven regenerated plants were NPT positive, whereas when kanamycin (25mg/l) was added to the embryo-inducing CTM-2 medium, some 40% of the regenerated plants were NPT-positive. Figure 2 shows the NPT test for 9 of the 11 plants, with Nos. 1 and 6 showing positive; No. 10 in Figure 2 is the positive control. The addition of 25 mg/l kanamycin did not affect the regeneration process of the transformed tissue, nor did it prevent regeneration of some NPT-negative plants. This concentration of kanamycin (25 mg/l) also allowed some growth of the control (non-tranformed) roots; however, the controls did not regenerate plants in the presence of kanamycin.

In another series of tests (Series C), kanamycin concentration was doubled to 50 mg/l, but this concentration of kanamycin slowed plant regeneration and increased the number of abnormal plantlets.

Southern blot analysis of the DNA from the NPT-positive plants (Figure 3) confirmed the integration of the vector T-DNA in cucumber DNA. Each transformed plant contained a single copy of foreign DNA, as indicated by the presence of two bands corresponding to two border fragments (Figure 1) resulting from the Hind III digest. DNA was isolated from five independent, NPT-positive plants, digested by Hind III and analyzed using Southern blots. The probes were pNEO 105 (vector DNA; Simpson et al., 1986), pFW94 or pFW41 (for T_L-DNA and T_R-DNA, respectively; Huffman et al., 1984). Among the five plants assayed for the integration of the Ri-plasmid DNA (which can be integrated into plant genome in two fragments; T left (T_L), and T right (T_R); White et al., 1985), two plants did not contain any Ri-plasmid T-DNA, one plant had a 5.7 kb fragment of the T_R-DNA, and two plants had different amounts of the T_L-DNA. This demonstrates that the method of this invention does not select strongly for or against T_L-DNA or T_R-DNA. Table II below summarizes the analysis proving the integration of the vector DNA as well as Ri plasmid DNA (both fragments), as follows:

TABLE II

Integration of the vector DNA (NPT) and the Ri-plasmid DNA (T_L and T_R) into DNA of five cucumber plants as a result of transformation with A. rhizogenes.

Plant #	A. Rhizogenes Vector Plasmid	Hind III fragments (kb) 1) Vector T-DNA (NPT)	T_L-DNA	T_R-DNA
1	pARC8	5.7; 4.9	0	5.7
2	pACR8	12.0; 4.8	0	0
3	pARC16	9.4; 4.9	0	0
4	pARC16	4/8; 3.9	3.4 1 5.9	0
5	pARC16	6.4; 3.7	3.4	

1) The probes were pNEO 105 (vector DNA; Simpson et al., 1986), pFW94 or pFW41 (T_L-DNA and T_R-DNA, respectively; Huffman et al., 1984)

2) This agrees with the mobility expected for the internal Hind III fragment (H-21) of the T_L-DNA, as described by White et al. (1985).

The tranfer of the vector-DNA only, vector plus Ri-DNA, or Ri-DNA only illustrates the flexibility of this system of transformation. The transfer of the vector-DNA only as observed in plants 2 and 3 (Table II) shows that a transfer of a desired gene can be achieved in cucumber using the A. rhizogenes strain without removing the Ri-DNA from the resident plasmid. In other words the resident Ri-plasmid does not have to be disarmed. On the other hand, since some of the characteristics associated with the Ri-plasmid DNA (like shorter internodes and male sterility) may also be desirable, Table II shows that cucumber plants having both the vector and the Ri-DNA can be created and selected. The method of transformation of this invention also permits recovery of plants containing only the RI-DNA, which plants thus will be compact and male sterile, if such characteristics only are desired.

Figure 4 shows a normal, control plant of C. sativus L., c.v. Straight Eight on the left, as compared to a transgenic plant of this invention, "PCRI HiDensity", on the right. Generally, the PCRI HIDensity plants may be described as trailing, annual herbs with branched hirsute vines and tendrils. Leaves are alternate, simple with petioles, palmately 3- to 5-lobed or angular, and thicker and narrower, pointed leaves may be expected in some plants. Tendrils are simple, lateral, stipuiar in position, one at each node. Transformed plants may be characterized from the control or wild plants in having shorter than normal internodes, on the order of 2.5 - 7.5 cm as compared to the normal 10 - 15 cm, resulting in a compact morphology. The flowers are monoecious, but gynoecious, perfect, andromonecious and trimonecious forms are also expected to occur. The flowers are yellow or cream colored, fascicled or solitary, and often borne at every node. The staminate flowers have the calyx and corolla united to form a tubular receptacle, stamens basically 5, alternate with petals, filaments free or united, anthers free or coherent in a head. The pistillate flowers have the calyx and corolla similar to the male flowers, ovary inferior usually with one locule and 3 or 4 carpels, style solitary with 2- or 3-lobed stigma. The fruit is a fleshy berry, indehiscent. The seeds are large, numerous, white or tan colored, with no endosperm, and embryos have large cotyledons. The seeds from the fruit of compact (short internodal) genetically transformed plants of this invention are germinable and have been grown into the R2 generation to date.

Upon maturation, the transgenic as well as the regenerated control cucumbers displayed varing degrees of diminution and abscission of male flowers. The abscission of male flowers in the control plants (which were

regenerated but not transformed indicates that the reduced fertility most likely did not result from transformation, es was reported in other species (Tepfer, 1984), but rather was due to abnormalities associated with the regeneration procedures. In heritable form, these genetic alterations are significant and beneficial in Cucumis (particularly C. sativus) breeding, since compact stature and male sterility are desirable in this species (Kauffman and Lower, 1976). Thus, shortened internodes developed in these plants permit production of the same fruit on lesser acreage, at correspondingly lower costs. Stated conversely, more fruit can be produced from the same acreage.

Surprisingly, none of the transgenic plants showed abnormalities in leaf morphology, such as leaf wrinkling as expected from the reports of tobacco, carrot and morning glory transformed with sA. rhizogenes (Tepfer, 1984).

Using the methods of this invention, we recovered transgenic cucumber plants within 10 weeks. This methodology is simpler and faster than the co-cultivation method (Marton et al., 1979) in which plant protoplasts are transformed. The co-cultivation method is labor-intensive and prone to contamination, whereas the techniques of this invention consist of a few, simple steps that can be effectively performed in standard laboratories. Furthermore, somaclonal variation frequently results from protoplast culture, whereas organized tissue is known to remain more stable (Shepard et al., 1980; Krens et al., 1982; Horsch et al., 1985).

Although our work indicates that some cucumber cultivars do not regenerate plants from roots under the specified conditions (Trulson and Shahin, 1986; or copending case 92/9), the cultivars discovered by us which are capable of root regeneration (Sunblest, Burpless, Bush Slicer, GY 14, Streight 8) can serve as intermediates in gene transfer to other genetic backgrounds. Additionally, since the genotypes capable of regeneration from roots represent highly advanced germplasm, genetic engineering performed in this germplasm will help to advance already superior genotypes.

The methods of transformation of this invention also facilitate somatic hybridization (including both hybrids and cybrids) in Cucumis sp. plants, particularly cucumber. Although the potential of somatic hybridization is well recognized, the previous lack of selectable markers constitutes a major obstacle in manipulating protoplasts of higher plants (Cocking et al., 1981. Thus, the methods of this invention permit the introduction of drug-resistance markers to aid in identification and selection of desirable protoplast (or p_x/c_y) fusion products.

Further, DNA encoding chloramphenicol-resistance can be transferred to chloroplasts (Van den Brock et al., 1985). Thus, with the methods and markers of this invention, kanamycin and chloramphenicol resistance can be introduced into plant tissue by appropriate vectors, and chloramphenical and kanamycin can be used to select for nucleus/chloroplast fusion products with the desired marked traits. Thus, somatic hybrid isolation and selection is facilitated. Moreover, these markers would be of great value in transfers of agronomically important genes, since the presence of an easily identifiable markar linked to an agronomically desirable gene would permit efficient selection in tissue culture (Fraley et al., 1983; Herrera-Estrella et al., 1983).

Exemple B. Transformation/Somatic Hybridization.

Somatic hybridization is accomplished by protoplest-protoplast or protoplast-cytoplest fusion (generically p_x/c_yfusion). The protoplasts and/or cytoplasts of different species, cultivars or genetically altered plants (such as the transgenic plants of this invention) having desired traits should be seperately liberated, prepared, fused and regenerated as described in our copending case 92/13.

Transformation may precede, or follow, somatic hybridization, or may be sequentially combined with electroporetion, cocultivation or chemical fusion techniques, and somaclonal variation selection via culturing, to assist in isoleting and selecting plants with desired traits.

Further, these fusion techniques can also be used to genetically transform protoplasts by fusing them with liposomes (phospholipid bilayer vesicles) that may contain foreign DNA, or bacterial sphereoplasts, which are bacterial cells devoid of cell walls.

Example C. Encepsulation ot Transgenic Embryoids -
Artifical Seeds. Since in vitro production of somatic embryoids es in Example A5 above results in embryoids without protective sead coats, the transgenic embryoids ot this invention may be encepsuleted, individually or in groups, in capsules or coatings to retard dehydration and preserve them for future "planting" and growing as set forth in our copending cases 92/9 or 92/13.

Exampla D. Regeneration After Freeze Storage of Transgenic
Embryoids/Mini-Calli. One or more of the transgenic protoplastt sources, protoplasts therefrom, mini-calli, or embryoids may be stored trozen for extended periods, then thewed and regenerated following the steps and media set torth in our copending cases 92/9 and 92/13.

It should be understood that various modifications within the scope ot this invention can be made by one of ordinery skill in the art without departing from the spirit thereof. For example, the positive response of Cucumis sp. plants to transformation and regeneration by the media and methods of this invention render them useful in breeding. One important application is in the production ot gynoecious populations to replace the current expansive treatment with silver nitrete. Another application ot this invention is use ot the techniques herein in combination with somatic hybridization and/or somaclonal variation as e veluable source of diversity in plant meterial used in breeding. The fusion of transtormed protoplasts or transformation ot p_x/c_y fusion, followed by

regeneration may be used for selection of individuals resistant to pathogens, toxic metals, pesticides and herbicides. The latter is of particular importance because cucumbers are very sensitive to herbicides.

The application of genetic engineering in cucumber and muskmelon breeding is of special value. These two species are sexually incompatible. Conventional crosses and transfer of many desirable traits like disease resistance heretofore have not been possible (Deakin et al., 1971). Such barriers are now removed with the development of capability of gene transfer through plant transformation disclosed herein, used alone or in combination with somatic hybridization disclosed in our copending case 92/13. Further, the explant regeneration culture techniques of our copending case 92/9 leads to selection of desirable traits through somaclonel variation.

In addition to the ability to transfer horticulturally desirable genes into cucumber or muskmelon, genetic engineering procedures are particularly valuable in introduction of marker genes such as resistance to kanamycin or chloramphenicol. These markers facilitate somatic hybridization via protoplast fusion, thus removing the sterility barriers in the genus Cucumis.

We therefore wish our invention to be defined by the scope of the appended claims as broadly as the prior art will permit, and in view of this specification if need be.

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Claims

1. The new and distinct veriety of cucumber plant, PCRI Hi-Density, shown and described, and tha seeds and embryoids or mini-calli thereof.
2. A method of transforming plant tissue which comprises
inoculating seid plant tissue with A. rhizoganes containing a vector in addition to the resident Ri plasmid for a time sufficient to introduce said vector to at least some of the cells of said tissue to form at least one transgenic cell, and
optionally culturing at least one of said transgenic cell(s) in an appropriate medium for a time sufficient to regenerate a competent plant structure selected from a competent colony of cells, tissue, mini-calli, embryoid, plantlet, plant and seed.
3. The method of claim 2 wherein said plant tissue is hypocotyl tissua.
4. The method of claim 2 wherein said plant tissue is from a member of Cucumis sp.
5. The method of claim 1 wherein said culturing step includas culturing on CTM-3 Medium, followed by culturing on CTM-3 to form a mature transgenic embryoid, and transferring said embryoid to CTM-4 Medium for development into a plantlet.
6. Competent transgenic mini-calli or embryoids produced by the process of claim 2.
7. A method of culturing competent transgenic mini-calli or embryoids produced by the method of claim 2 and frozen which comprises
thawing said frozen transgenic embryoid or mini-calli; and
culturing said thawed transgenic embryoid or mini-calli to promote further development and maturation
8. A method of obtaining a transgenic plant tissue which comprises the steps of:
a) fusing one ore more cellular-derived material(s) selected from one or more protoplast(s) , cytoplast(s) , sphereoplast(s) , liposome(s) and mixtures thereof, from one recipient plant, with one or more cellular-derived material selected from one ore mora protoplast(s) , sphereoplest(s) , liposome(s) , cytoplasts and mixtures thereof from another, donor organism to produce a fusion product; and
b) culturing seid fusion product for a time sufficient to produce a viable genetically altered mini-calli or embryoid.
9. The method of claim 8 wherein said fusion is selected from p_x/c_y fusion, and said donor material source is selected from plant and bacterial cell material.
10. The method of claim 9 wherein said materiel source for both said donor and racipient is from Cucumis sp. plant cells.

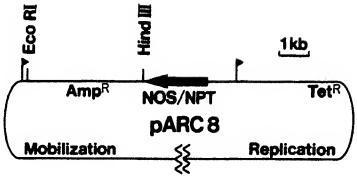
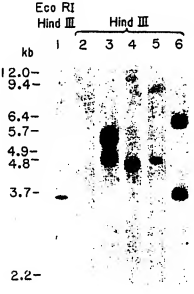


Fig. 1.

Fig. 3.



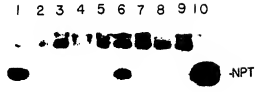


Fig. 2.

Fig. 4.

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(54) **Genetic transformation and controlled regeneration of cucumis SP in vitro.**

(57) Transformation of Cucumis sp. plants with A. rhizogenes followed by regeneration into genetically altered plants. Inverted hypocotyls of C. sativus L., cv. Straight Eight were inoculated with A. rhizogenes containing the vector pARC8 or pARC16 containing NOS/NPT chimeric marker gene conferring resistance to kanamycin in addition to the resident Ri plasmid. Roots produced as the inoculated hypocotyls were excised and sequentially regenerated in CTM-2, -3 and -4 media, resulting in mature, fruiting transgenic plants having germinable seed. Short internodal morphology was expressed yielding Cucumber plants, called "PCRI HI Density", having greater yield per acre.

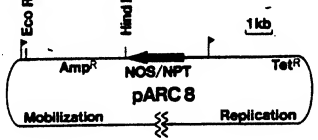

Fig. 1.

EP 0 262 972 A3

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number
EP 87 30 8720

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
O,X	UCLA SYMPOSIUM NEW. SER., vol. 44, Tahoe City, 22nd-28th February 1986, Molecular Biology of Plant Growth Control, pages 381-389, A.R. Liss, Inc; K. SUKHAPINDA et al.: "Phenotype of plants derived from hairy roots transformed with Agrobacterium rhizogenes" * Page 382, last paragraph; page 383, last paragraph; page 386, paragraph 3 *	2,4	A 01 H 1/00 C 12 N 15/00 C 12 N 5/00 A 01 G 7/00
X	THEOR. APPL. GENET., vol. 72, no. 6, September 1986, pages 770-777, Springer-Verlag; E.A. SHAHIN et al.: "Transformation of cultivated tomato by a binary vector in Agrobacterium rhizogenes: transgenic plants with normal phenotypes harbor binary vector T-DNA, but no Ri-plasmid T-DNA"		

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

A 01 H
C 12 N
A 01 G

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely: 2-7
Claims searched incompletely:
Claims not searched: 1
Reason for the limitation of the search:

Art. 53b. Plant variety.

Place of search	Date of completion of the search	Examiner
THE HAGUe	01-06-1990	MADDOX

EPO Form 1503.1 03.82

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y	* Whole document * --	3,4,6	
O,Y	COMMONWEALTH AGRICULTURAL BUREAU, abstract no. 88167099; M.J. ONDREJ et al.: "Plant regeneration from Agrobacterium rhizogenes induced tumors" & INST. EXP. BOT., ACAD. SCI., Na Sadkach 702, 370 05		
	* Abstract * --	4	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Y	MOL. GEN. GENET., vol. 202, no. 3, March 1986, pages 388-393, Springer Verlag; A. PETIT et al.: "Multiple transformation of plant cells by Agrobacterium may be responsible for the complex organization of T-DNA in crown gall and hairy root"		
	* Page 399, left-hand column, paragraph 5 * --	3	
Y	NATURE, vol. 321, 12th June 1986, pages 669-674; J. STOUGAARD JENSEN et al.: "Nodule-specific expression of a chimaeric soybean leghaemoglobin gene in transgenic Lotus corniculatus"		
	* Page 669, right-hand column, paragraph 2 * --	6	
A	PLANT MOLECULAR BIOLOGY, vol. 6, no. 6, 1986, pages 403-415, M. Nijhoff Publishers, Dordrecht, NL; R.B. SIMPSON et al.: "A disarmed binary vector from Agrobacterium tumefaciens functions in Agrobacterium rhizogenes"		
	* Whole article * -----	2-7	

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EXHIBIT B

Plant Molecular Biology 6: 403–415, 1986
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A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes*

Frequent co-transformation of two distinct T-DNAs

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Keywords: *Agrobacterium tumefaciens, A. rhizogenes*, hairy root, plant, transformation, vector

Summary

Binary Ti plasmid vector systems consist of two plasmids in *Agrobacterium*, where one plasmid contains the DNA that can be transferred to plant cells and the other contains the virulence (*vir*) genes which are necessary for the DNA transfer but are not themselves stably transferred. We have constructed two non-oncogenic vectors (pARC4 and pARC8) based on the binary Ti plasmid system of *Agrobacterium tumefaciens* for plant transformation. Each vector contains the left and right termini sequences from pTiT37. These sequences, which determine the extent of DNA transferred to plant cells, flank unique restriction enzyme sites and a marker gene that functions in the plant (nopaline synthase in pARC4 or neomycin phosphotransferase in pARC8). After construction *in vitro*, the vectors can be conjugatively transferred from *E. coli* to any of several *Agrobacterium* strains containing *vir* genes. Using *A. rhizogenes* strain A4 containing the resident Ri plasmid plus a vector with the nopaline synthase marker, we found that up to 50% of the hairy roots resulting from the infection of alfalfa or tomato synthesized nopaline. Thus, vector DNA encoding an unselected marker was frequently co-transferred with Ri plasmid DNA to an alfalfa or a tomato cell. In contrast, the frequency of co-transfer to soybean cells was difficult to estimate because we encountered a high background of non-transformed roots using this species. Up to five copies of the vector DNA between the termini sequences were faithfully transferred and maintained in most cases suggesting that the termini sequences and the *vir* genes from the Ri and Ti plasmids are functionally equivalent.

Introduction

Because of their natural ability to transfer DNA to plant cells, *Agrobacterium tumefaciens* and its Ti plasmids have been used as vectors to introduce foreign DNA into plants (as reviewed recently 27, 34, 54). Since the production of auxins and cytokinins by transformed cells is often incompatible with normal plant regeneration, it is frequently desirable to 'disarm' the plasmids by removing the oncogenes responsible for the synthesis of these growth regulators and to introduce selectable or screenable markers in their place. The large size of the Ti plasmids makes it necessary to use intermediate vectors. One intermediate vector method is the 'co-integration' approach, whereby foreign DNA is inserted into a vector that cannot replicate in *Agrobacterium*, but can recombine with the Ti plasmid through a homologous portion of the vector, producing a co-integrate of the two plasmids (16, 21, 55). Another method is the binary vector approach whereby a foreign gene is inserted into a disarmed T-DNA which itself is joined to a broad host range replicon that can replicate in *Agrobacterium* (3, 6, 17, 24, 26, 29).

The mechanism of T-DNA transfer from the bacteria to the plant is not known in detail, but at a minimum, transfer requires termini sequences and *vir* genes from the Ti plasmid in addition to bacterial chromosomal genes. The termini sequences

are imperfect 25 basepair direct repeats found flanking the T-DNA, at least one of which is required for the transfer (9). The *vir* genes are required for DNA transfer but are themselves not stably transferred to the plant (34). Although the *vir* genes and the T-DNA are normally part of the same bacterial replicon, a binary system is possible, in which these functions are on separate replicons (17, 24, 26).

Since an oncogenic marker is not present in these disarmed vectors, other markers have to be used to identify genetically transformed plant cells. Some markers, such as enzymes which result in the production of opines, can be used to screen transformed tissue for the presence of opines (e.g., octopine, nopaline or agropine; 37). Other markers, such as enzymes which confer resistance to antibiotics, can be used to select transformed tissue which can grow in the presence of an antibiotic (e.g., kanamycin, chloramphenicol or methotrexate; 23).

Agrobacterium rhizogenes (27) is considered a close relative of *A. tumefaciens* because of its similar mechanism of plant transformation based on DNA transfer to plants, the similar function of the *vir* genes, and the production of opines by transformed tissue. *A. rhizogenes* frequently produces transformed, hairy roots so the endogenous plasmid has been called the Ri ('root-inducing') plasmid. Hairy roots from several species have been regenerated into plants which contain T-DNA from the Ri plasmid (10, 12, 43, 44). In cases where plants can be regenerated from roots, the combination of *vir* genes from the Ri plasmid and a gene transfer vector derived from the T-DNA of *A. tumefaciens* may be the system of choice for gene transfer.

We report here, the construction of binary vectors for use in *A. tumefaciens* or *A. rhizogenes* containing either, a selectable marker that confers kanamycin-resistance to transformed plant cells, or a marker that is easy to screen, nopaline synthase. Inoculation of several plant species with *A. rhizogenes* containing a vector resulted in hairy roots. With alfalfa and tomato, we could demonstrate frequent co-transfer of vector DNA and Ri plasmid DNA. Southern analysis of the roots has shown that in most cases the DNA was transferred and integrated faithfully into the plant genome.

Materials and methods

The procedure for transformation of *Escherichia coli* with plasmid DNAs is as described (1). Other manipulations of nucleic acids are essentially those described by Maniatis *et al.* (32), unless otherwise indicated. Restriction enzymes and pUC8 were obtained from Bethesda Research Laboratories. The BglII linkers were from New England Biolabs. The bacterial strains and plasmids used for these experiments are listed in Table 1.

Plasmid constructions

The vector pARC4 was constructed as illustrated in Fig. 1A. The plasmid pBstEII 9, 14 (52) carries the 1.5 kb EcoRI fragment 29 derived from Ti plasmid pTiT37. This fragment contains the left terminus of the T-DNA region located approximately 50 basepairs from the right end of the fragment (51, 53). The plasmid pT37H23, a generous gift from Scott Stachel, carries the 3.2 kb HindIII fragment 23 from Ti plasmid pTiT37 (18). From left to right, this fragment contains the 5' portion of the DNA encoding transcript 6b, the entire nopaline synthase or *NOS* gene, the right terminus sequence and a portion of the Ti plasmid which is not transferred to plant cells.

The chimeric gene '*NOS/NPT*' was constructed by placing the coding region for the neomycin phosphotransferase II gene (*NPT*) from the bacterial transposon Tn5 under control of the transcriptional regulatory signals of the nopaline synthase gene (*NOS*). The '*NOS/NPT*' gene is part of the plasmid pNEO105 whose structure is shown schematically in the bottom panel of Fig. 2. The *NOS* gene fragment was derived from the plasmid pT37H23 (18). Based on the numbering convention of Depicker *et al.* (18), pNEO105 contains the nopaline synthase promotor (from the BclI site at position −265 to position +30) and polyadenylation site (from the SphI site at position +1136 to the HindIII site at position + 1972). The *NPT* fragment was derived from the plasmid pNEO (P-L Biochemicals) which contains the neomycin phosphotransferase II gene from Tn5 cloned into pBR322. Based on the numbering convention of Beck *et al.* (4), pNEO105 contains a portion of the *NPT* gene (from position 1543 to the SmaI site at

Table 1.

Plasmids name	Description	Reference
pBstEII 9, 14	pTiT37 BstEII fragments 9 & 14 cloned in pMB9	Yang & Simpson (52)
pUC8	AmpR	Vieira & Messing (47)
pT37H23	AmpR; pTiT37 HindIII fragment 23 cloned into pBR322	Depicker *et al.* (18)
pUC8-22-21	AmpR; pTiT37 EcoRI fragment 29 cloned into pUC8	This paper.
pRK290	IncP; TetR, derivative of pRK2	Ditta *et al.* (19)
pRK2013	IncP; KanR; can mobilize pRK290 and its derivatives	Ditta *et al.* (19)
pARC1	AmpR; pTiT37 HindIII fragment 23 cloned into pUC8-22-21	This paper.
pRKM1	pRK290 with EcoRI site eliminated	This paper.
pARC3	Deletion derivative of pARC1	This paper.
pARC4	pARC3 cloned into BglII site of pRKM1	This paper.
pNEO	*NPT*II gene from Tn5 cloned into pBR322	P-L Biochemicals.
pNEO105	Nos/*NPT* gene in pBR322	This paper.
pARC8	Replacement of Nos gene in pARC4 with *NOS/NPT* gene	This paper.
pRUD26	Chimeric soybean *SS/NPT* gene in pARC4	McKnight & Simpson, in preparation.
pRUD27	Chimeric soybean *SS/NPT* gene in pARC4	McKnight & Simpson, in preparation.
pSW94	Left-T-DNA of pRiA4	Huffman *et al.* (28)
Escherichia coli strains		
HB101	F-, hsdS20(rB-mB-), recA 13, ara-14, proA2 lacY1, galK2, rpsL20 (SMR), xyl-5, mtl-1, supE44, lambda	Boyer and Roulland-Dussoix (8)
JM83	ara, del lac-pro, strA, thi, Phi80dlacZ del M15	Vieira & Messing (47)
Agrobacterium strains		
A4	*Agrobacterium rhizogenes;* pRiA4	White and Nester (48)

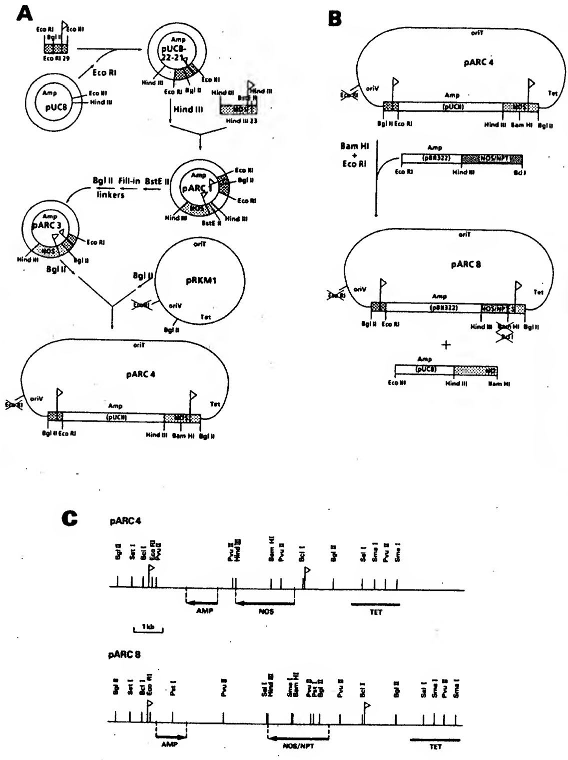
position 2516), which includes the entire coding region from the *NPT* gene. The vector pARC8 was constructed from pNEO105 and pARC4 as illustrated in Fig. 1B.

Introduction into Agrobacterium

The vectors were transferred from *E. coli* to *A. rhizogenes* A4 by conjugation, in the presence of a third bacterium, *E. coli* strain HB101 containing the plasmid pRK2013 (19) to mobilize the vectors. Growth on AB minimal sucrose medium containing biotin (49), plus 5 μg/ml tetracycline selects for *Agrobacteria* containing the vector. Furthermore, the use of minimal medium without added amino acids selects against *E. coli* amino acid auxotrophs such as HB101.

Inoculation and establishment of hairy root cultures

Plant material: Stems from tobacco (*Nicotiana*



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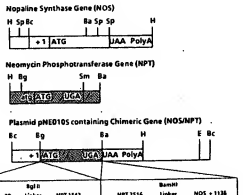


Fig. 2. Schematic drawings of the nopaline synthase gene (*NOS*; represented by the white bar) from the T37 Ti plasmid (first panel), the neomycin phosphotransferase gene (*NPT*; represented by striped bar) from Tn5 (second panel), and the plasmid pNEO105 containing the chimeric gene *NOS/NPT* (third panel) where pBR322 sequences are represented by the thin line. Plasmid pNEO105 is shown linearized at the BclI site. The drawings are to indicate relative positions only and are not to scale. The following symbols are used for orientation: start-site of transcription (+1), startsite of translation (ATG), site of translational termination (UAA or UGA), and site of transcriptional termination (polyA). The 'atg' refers to a region of the *NPT* gene containing an ATG codon (other than the initiation ATG) that we did not include in the *NOS/NPT* gene. Bevan (6) and Fraley *et al.* (21) report that the elimination of this 'atg' in a chimeric gene construction similar to *NOS/NPT* results in a higher level of resistance to kanamycin. The symbols for restriction enzymes sites discussed in the text are the following: BamHI (Ba), BclI (Bc), BglII (Bg), EcoRI (E), HindIII (H), SmaI (Sm), and SphI (Sp). At the bottom are the DNA sequences in the vicinity of the BglII and BamHI linkers in the *NOS/NPT* gene. The numbers refer to the numbering system of Depicker *et al.* (18) for the *NOS* gene and to the numbering convention of Beck *et al.* (4) for the *NPT* gene. Note that the *NOS/NPT* gene is drawn in the opposite orientation to that in Fig. 1.

tabacum, cv. *Xanthi*), tomato (*Lycopersicum esculentum*, cv. red cherry), soybean (Glycine max cv. Williams 82) and alfalfa (*Medicago sativa* cv. CUF101) plants grown in a greenhouse were sterilized by soaking in 0.5% $Ca(OCl)_2$ + 0.25% Tween 80 for 30–60 min and then washed 3–4 times with sterile distilled water. The stems were cut in sections (2–3 cm), inverted and transferred aseptically to solid hormone-free TM-1 medium (40) + 250 mg/l cefoxitin (Merck, Sharp and Dohme) or recently, cefotaxime (Calbiochem) in plastic boxes. Some of the *Agrobacterium* strains are resistant to ampicillin and to Carbenicillin but not to cefoxitin (11) or cefotaxime (23). Over a period of 6 months, a minimum of 5 individual plants of each species were used. A bacterial suspension in stationary phase, taken from either solid or liquid selective medium containing tetracycline, was spread on the upper surface of the inverted stem section. The inoculated stem sections were incubated at 25 °C in low light with a 16 hr photoperiod. Roots emerging from the top of the stem section after 2–3 weeks were excised several days later when about 1 cm long and transferred to the same medium. They were grown in the dark at 25 °C and subcultured every 3 to 4 weeks. In order to select for kanamycin-resistant hairy roots, roots were transferred to hormone-free TM4- medium (40) containing cefotaxime (300 mg/l) and kanamycin (20–30 mg/l for alfalfa, 30–50 mg/l for tomato, 25–30 mg/l for tobacco, and 20–100 mg/l for soybean). After 2 weeks roots which were still growing were considered to be kanamycin resistant. Higher levels of kanamycin in this assay slowed the growth of all roots, even those expressing high levels of NPT, while lower levels of

Fig. 1. Schematic drawings of the steps in the construction of vectors pARC4 (panel A) and pARC8 (panel B). Fragments from pTiT37 containing the left terminus sequence (EcoRI 29) and the nopaline synthase gene plus right terminus sequence (HindII 23) were sequentially cloned into pUC8 creating pUC8-22-21 and then pARC1. To eliminate a HindIII site and an EcoRI site in pARC1, it was digested with BstEII, the ends were filled in, a BglII linker was inserted, and it was cut with BglI and religated to form pARC3. After linearization with BglII, pARC3 was inserted into the BglII site of pRKM1 (a derivative of pRK290 without an EcoRI site) to create the binary vector pARC4. The binary vector pARC8 was created by the replacement of the 'small' EcoRI-BamHI fragment from PARC4 with the 'large' EcoRI-BclI fragment of pNEO105. Note that the the *NOS/NPT* gene is drawn in the opposite orientation to that in Fig. 2. The symbols used for regions of DNA are the following: bacterial genes for resistance to tetracycline (Tet) and ampicillin (Amp); origin of vegetative DNA replication (oriV); origin of conjugative transfer (oriT); pBR322 or PUC8 sequences (open bar); pTiT37 EcoRI fragment 29 (bar with large dots); pTiT37 Hind fragment 23 (bar with small dots); pRK290 sequences (thin line); right and left termini sequences (flags); pTiT37 nopaline synthase gene (*NOS*); *NOS/NPT* gene (striped bar). The location of restriction enzyme sites discussed in the text are indicated. Where the name has been crossed out, the restriction site has been eliminated. The vector pARC4 is about 26 kb including a transferred DNA of about 5 kb while pARC8 is about 28 kb including a transferred DNA of about 7 kb. Panel C, which is drawn to scale, contains detailed restriction enzyme maps of the region around the transferred DNA in pARC4 and pARC8.

kanamycin permitted some roots without a NPT gene to continue growing for 2 weeks. Kanamycin-resistant roots all contained *NPT* II activity based on the native gel assay of Reiss *et al.* (39). To increase the quantity of tissue rapidly, we transferred the roots to the same medium without kanamycin. Prior to DNA isolation, roots were propagated without cefotaxime to verify that they were free of bacteria.

In preparation for hypocotyl infections, seeds were sterilized by soaking in 70% ethanol for 2 minutes, then in 20% of commercial bleach for 30 minutes and finally washed 3–4 times with sterile distilled water. The seeds were germinated on TM-1 medium in plastic boxes. Seedlings were infected with *Agrobacterium* after 2–3 weeks by wounding the hypocotyl with a needle tip covered with stationary-phase bacteria. The roots were then handled as described above.

Nopaline assay and DNA analysis

For the nopaline assay, tissue (20–100 mg) in a 1.5 ml Eppendorf tube was homogenized using a wooden applicator stick. The debris were pelleted for 5 min in a Brinkman Eppendorf microcentrifuge. Up to 50 μl of supernatant was pipetted onto 5 mm filter paper discs (Whatman #3). The discs were air-dried and then placed at the origin prior to high voltage paper electrophoresis and staining essentially as described by Otten and Schilperoort (37). For DNA analysis, gels were prepared for Southern transfer and hybridization essentially as described in Thomashow *et al.* (45).

Results

Construction of the binary vector pARC4

Figure 1A illustrates the steps in the construction of our first vector, pARC4. For the purpose of DNA manipulations in bacteria, the vector contains a wide host range bacterial replicon, a bacterial origin of transfer, a bacterial antibiotic resistance marker, and unique restriction enzyme sites. The wide host range origin of replication (oriV), derived from the wide host range plasmid RK2 (19), permits replication of the plasmid in both *Escherichia coli* and in *Agrobacterium*. In contrast, pBR322 and ColE1 replicons are unable to replicate in *Agrobacterium*. The origin of transfer (oriT), also from plasmid RK2, permits the vector to be mobilized by a helper plasmid; mobilization is the most efficient means to introduce plasmids into *Agrobacterium*. The antibiotic marker, bacterial resistance to tetracycline (Tet) from plasmid RK2, allows selection of bacteria containing the vector. The unique restriction enzyme sites, EcoRI and HindIII, facilitate the insertion *in vitro* of 'foreign' DNA fragments into the vector.

For the purpose of transferring DNA to plant cells, pARC4 contains the signals (termini sequences) which *Agrobacterium* uses to delimit the DNA it transfers to plant cells – the foreign DNA is placed between these two signals. The termini sequences, denoted by flags in Fig. I, are both derived from pTiT37, the left from EcoRI fragment 29 and the right from Hind III fragment 23 (51, 53). Although, in some cases, a single terminus region has been shown to be all that is absolutely required for transfer (9), none of the resulting transfers have been characterized. Both termini sequences were therefore used to increase the likelihood of predictable and reliable transfer. To identify plant cells that contain vector DNA, pARC4 contains the nopaline synthase gene (*NOS*) from the T37Ti plasmid as part of the transferred DNA. A rapid assay of plant cells can identify those which synthesize nopaline and thus contain the transferred DNA.

In addition to these essential components, the vector contains within the transferred DNA the bacterial ampicillin-resistance gene (*Amp*) and the narrow host range ColE1 origin of replication derived from pUC8. These could prove useful in rescuing the transferred DNA from the plant subsequent to transformation. Also, during some manipulations with the vector, such as the replacement of the small EcoRI/HindIII fragment in pARC4 with a foreign gene, 'Ampscreen' (Bethesda Research Laboratories) has been used to screen for the loss of ampicillin resistance. Finally, the addition of the CoLE1 origin of replication permits one to amplify the plasmid in *E. coli* (but not in *Agrobacteria*). This facilitates rapid, small-scale plasmid isolations and characterizations, as well as the initial preparation of the vector.

Table 2. Transfer of nopaline synthesis and kanamycin resistance markers into roots resulting from infection of 4 plant species with *A. rhizogenes* containing pARC4 type or pARC8 type vectors.

Plant system	Proportion of independent roots†	
	Nopaline Positive (pARC4 Type)	Kanamycin Resistant (pARC8 Type)
Alfalfa		
Stem Section	63/115 (55%)	NT*
Hypocotyl	14/43 (33%)	2/40 (4%)
Tomato		
Hypocotyl	8/24 (33%)	18/95 (19%)
Tobacco		
Stem Section	NT	16/90 (18%)
Soybean		
Stem Section	1/58 (2%)	0/80 (0%)
Hypocotyl	2/108 (2%)	NT

† As discussed in the text, the alfalfa, tomato and tobacco roots were hairy roots while the majority of the soybean roots were untransformed.
* NT – Not Tested.

had no apparent effect on the results, we report the data grouped by type of vector. The results include pARC4, pARC4 containing 8 different inserts ranging in size from 3 kb to 7 kb, pARC8, and pARC8 containing a 4 kb insert or a 7 kb insert. Only pARC8 was used with all four species.

Surprisingly, over 50% of the roots, derived from the infection of alfalfa stem sections with A4 containing pARC4 or a pARC4 derivative, synthesized nopaline. The percentage of nopaline positive roots in pARC4 experiments was higher than the percentage of kanamycin resistant roots in pARC8 experiments using either alfalfa hypocotyls, tomato hypocotyls or soybean stem sections. Since the nopaline assay was a screen and the kanamycin resistance was used as a selection, one explanation is that the level of kanamycin used for selection was too high for some transformed roots to survive. Kanamycin slowed the growth of hairy roots in our experiment, even those expressing high levels of *NPT*. Also, kanamycin promotes an alternative developmental pathway, shooting, in tobacco and carrot (38). Another explanation is that DNA transfer from pARC4 may be more efficient than DNA transfer from pARC8.

Construction of the selectable marker 'NOS/NPT' and vector pARC8

Shown in Fig. 2 is the structure of the plasmid pNEO105 which contains the chimeric gene *'NOS/NPT,* a selectable marker for transformed plant cells. The *'NOS/NPT* gene consists of the promotor and transcriptional termination site of the nopaline synthase gene (*NOS*) from pTiT37 and the coding region from the neomycin phosphotransferase II (*NPT*) from the bacterial transposon Tn5.

As illustrated in Fig. 1B, the vector pARC8 is similar to pARC4 except that the *NOS* marker is replaced by the *NOS/NPT* marker, which functions in plant cells to produce the enzyme neomycin phosphotransferase II. Plant cells containing the enzyme are resistant to kanamycin. Further, the presence of the enzymatic activity can be assayed directly (39).

Introduction into Agrobacterium

The vectors pARC4, pARC8 and their derivatives were transferred by conjugation to several *Agrobacterium* strains including A4 (48) and LBA4404 (36). In 2 out of 68 occasions, we found that a vector construction had a different structure in *Agrobacterium* than it had in *E. coli.* Klee *et al.* (29) have also observed an alteration in the structure of a binary vector after transfer from *E. coli* to *Agrobacterium.*

Plant transformation

Using *Agrobacterium rhizogenes* strain A4, containing either the nopaline (pARC4) type vector or the *NOS/NPT* (pARC8) type vector, we inoculated inverted stems or hypocotyls of tobacco, tomato, alfalfa and soybean. The resulting roots were excised and transferred to hormone-free media and grown as separate root clones. Neither uninoculated tissue nor tissue inoculated with a disarmed *A. tumefaciens* strain (LBA4404, ref. 36) produced roots with tomato, tobacco or alfalfa. In contrast, such controls frequently produced roots with soybean. Table 2 summarizes the results of assays on the roots from each of the four species either for the presence of nopaline or for ability to grow in the presence of kanamycin. Since the type of insert

Analysis of DNA from transformed plant cells

DNA was isolated from roots, digested with one of several different restriction enzymes, fractionated by gel electrophoresis and transferred to nitrocellulose. The resulting Southern blots were probed with radiolabelled DNA corresponding to portions of the vectors.

If there were faithful transfer of DNA to the plant cell, we would expect all of the DNA between the termini sequences to have been transferred. Examples of faithful and aberrant transferred copies are shown in Fig. 3. Constructs pRUD26 and pRUD27 contain two different versions of a chimeric gene inserted into pARC4 (McKnight and Simpson, in preparation). The chimeric genes are composed of portions of a gene from soybean (SRS 2.1 from ref. 5) encoding the small subunit of ribulose bisphosphate carboxylase (SS) and the coding region of the neomycin phosphotransferase gene (*NPT*, ref. 4). The probe and the hybrid SS/*NPT* genes were derived from a 1.5 kb soybean small subunit promotor fragment. Lane 3, which contains DNA from untransformed soybean leaves, has a 1.5 kb band representing the endogenous small subunit fragment. This band is also visible in the other lanes containing soybean DNA. The soybean hairy root lines A2 and M3 resulted from infection by A4(pRUD26). The DNA from line A2 (lane 4) shows a band of hybridization of about single copy intensity with the same mobility as the 3.6 kb band in the reconstructions (lanes 1 and 2). This suggests that there has been a faithful transfer of this fragment from pRUD26 to the soybean genome. DNA from the soybean hairy root line M3 in lane 5, shows several bands which hybridize to the probe. These bands are present at near single copy levels, but none are of the expected size suggesting that they are aberrant copies. Lanes 6 and 7, contain DNA from two independent soybean hairy root lines transformed by A4(pRUD27). In both lanes a single copy, full length band can be seen with a mobility of 2.4 kb. In addition to this faithfully transferred fragment, the DNA of line K1 in lane 6 has a fragment of higher molecular weight. One likely explanation for this larger size is that transfer of the T-DNA stopped short of the EcoRI site which is near the left termini sequence of pARC4 (see Fig. 1) and thus the copy is an aberrant one. This larger fragment presumably ends at an EcoRI site near the integration site in the soybean genome.

Fig. 4 summarizes our conclusions about the copy number and structural integrity of the DNA in 12 root lines from the infection of soybean, alfalfa and tobacco based on Southern blot analyses. For each of the lines analyzed, the copy number was estimated by comparing the intensity of the hybridization signal to the reconstructions, in addition to the size and number of non-internal (border) fragments derived, for example, from restriction with EcoRI or HindIII alone (data not shown). The figure shows the number of root lines with the indicated approximate copy number.

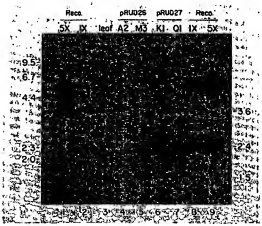


Fig. 3. Southern blot analysis of DNA from soybean hairy roots incited by A4(pRUD26) or A4(pRUD27).
The DNA in each lane was digested with both EcoR1 and HindIII, electrophoresed, blotted and hybridized with nick-translated pK26 which contains 1.5 kb of soybean DNA representing the SRS 2.1 small subunit promotor (McKnight, T., D. and Simpson, R. B., unpublished). Lanes 1 and 2 show 5 copy- and 1 copy-per haploid genome reconstructions for pRUD26. The lower band in these lanes indicates the expected size (3.6 kb) for a faithful, full length transfer of this fragment to the plant genome. Lane 3 contains DNA from untransformed soybean leaves and shows the 1.5 kb band representing the endogenous soybean small subunit promotor fragment from which the probe was derived. Lanes 4 and 5 represent DNA from 2 independent soybean hairy root lines (A2 and M3) arising from infection with A4(pRUD26). Lanes 6 and 7 represent DNA from 2 independent soybean hairy root lines (K1 and Q1) arising from infection with A4(pRUD27). Lanes 8 and 9 show 5 copy- and 1 copy-per haploid genome reconstructions for pRUD27. The lower band in these lanes indicates the expected size (2.4 kb) for a faithful, full length transfer of this fragment to the plant genome.

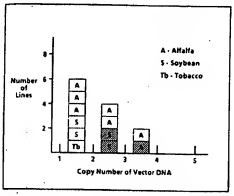


Fig. 4. Copy number and structural integrity of vector DNA found in transformed roots.
Summary of the structural analysis of DNA from 12 root cultures initiated by *Agrobacterium rhizogenes* A4 containing the vectors pARC4, pARC8 or one of these vectors with an insert. Each culture is represented by a box where the species is as indicated. The approximate number of copies of the DNA transferred from the vector is based on Southern blot analyses. A box with hatching denotes that one or more of the copies was 'aberrant' as described in the text.

Fig. 4 also illustrates which lines contained at least one 'aberrant copy' as indicated by the presence of bands on the Southern blot other than the expected internal fragments when an internal probe was used. The approximate copy number of the vector DNA in these lines varied from one to four. Of the 12 lines, 9 contained the expected portion of the vector DNA.

The Ri plasmid from *A. rhizogenes* strain A4 contains two separate DNAs that can be transferred to plant cells. The presence of the left T-DNA in 7 independent alfalfa root lines was examined by probing with pFW94 (28). In each case, transfer of the left T-DNA was indicated by the presence of bands with the mobilities expected of the 4.2, 3.4, 1.8 and 1.6 kb HindIII fragments internal to the left T-DNA (28), as well as other bands that probably represent border fragments.

Discussion

We have constructed two binary, disarmed vectors based on the Ti plasmid of *Agrobacterium tumefaciens*. Each vector, in several different *Agrobacterium* strains (this work and unpublished data), transfers DNA to plants. As described in the Results section, the vectors contain several features which allow them to be easily manipulated. Also, for the identification of transformed plant cells, one vector (pARC4) contains the nopaline synthase (*NOS*) marker while the other (pARC8) contains a chimeric gene composed of regulator signals from the *NOS* gene driving the expression of the neomycin phosphotransferase marker (*NOS/NPT*).

The use of binary vectors introduces additional flexibility to plant transformation approaches. Once a vector construction is complete, it can be used without modification in any of several different *Agrobacterium* strains including LBA4404 (unpublished work; 3, 6, 29), an *Agrobacterium* mutant containing the functional *vir* genes but no T-DNA (36). Our use of binary vectors in *Agrobacterium rhizogenes* permits the unusual regeneration potential of hairy roots (10, 12, 43, 44) to be exploited in conjunction with an efficient vector system.

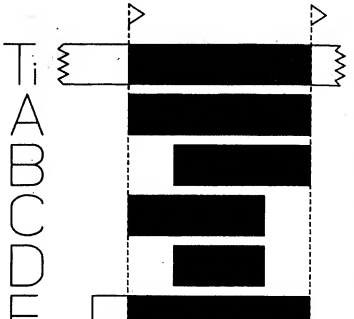
The choice of plant species inoculated made a considerable difference to the proportion of roots that were positive for the vector marker nopaline synthase (Table 2). It was technically easy to isolate a reasonable number of hairy roots that contain the appropriate marker using alfalfa, tomato or tobacco where up to half of the roots assayed were positive. However, to find positive soybean roots perhaps ten times more roots must be assayed since only a few percent of the soybean roots arising from the infection site were hairy roots. Perhaps even a high percentage of soybean roots which were hairy roots, synthesized nopaline. This conjecture is based on the high background production of soybean roots in the absence of *Agrobacteria* and on the observation that nopaline-containing roots had more lateral roots and an increased growth rate compared to normal soybean roots.

Each of the seven nopaline positive roots of alfalfa examined by Southern blot analysis contained both vector DNA and Ri plasmid DNA, confirming that the roots were in fact transformed, hairy roots. Thus, there was a high frequency of co-transfer to a plant cell of two distinct T-DNAs which originate on two separate bacterial replicons. Binary vectors have been derived from two different broad host range plasmids (pR772 and pRK2), the vectors contain termini sequences from either an octopine Ti plasmid (pTiAch5 or pTiA6) or a

nopaline Ti plasmid (pTiT37), and the vectors function in *Agrobacteria* containing any one of four families of Ti/Ri plasmids (3, 6, 17, 24, 25, 29). Indeed, T-DNA can be transferred to the plant even when moved to the bacterial chromosome while the *vir* genes remain plasmid borne (26). Thus, it is unlikely that the two plasmids must be a co-integrate to transfer DNA from the vector (17, 24). The transformed roots are organ clones and also probably cellular clones (12) suggesting that plant cells frequently can take up a second unlinked marker in addition to the first, selected marker.

The data at this point do not show a significant difference in the number of copies of vector T-DNA per plant cell using the two different vector approaches. The range reported in this paper (Fig. 4) for a binary vector, approximately 1–5, is consistent with the range for transformed plants derived from another binary vector (29) and from co-integration vectors (13, 16, 20, 21, 55). However, 5–20 copies have been reported in plants derived from a binary vector (6, 7, 29) and from a co-integration vector (14).

The data summarized in Fig. 4 indicate that 9 of 12 independently isolated root cultures contain the portion of the vector DNA that stretches from the left terminus sequence to the right terminus sequence. In each case, we ascertained the presence of an EcoRI site which lies only about 50 basepairs inside the left terminus sequence. It is thus a very sen-



Species	Tumor Line	T-DNA
Tobacco	A6S/2	A
Tobacco	B6806/E9	A
Tobacco	Tu-B6S3	A
Tobacco	WB-B6S3	A
Tobacco	SR1-B6S3	A
Tobacco	WB-A6	A
Tobacco	4013-1	A
Tobacco	4013-2	A
Tobacco	4001	A
Tobacco	4229	A
Alfalfa	Vertus ACH5	A
Alfalfa	Vertus B6 oct +	A
Sunflower	PSCG15955	A
Arabidopsis	A-Ach5	A
Tobacco	*W38C58-1	A
Tobacco	*BT37	A
Tobacco	LBA4013-4	B
Tobacco	Bla4	ABB
Tobacco	15955/1	C
Tobacco	A277/5	C
Alfalfa	Vertus B6 oct-	C
Petunia	P-Ach5	C
Tobacco	1595501	ABC
Flax	*FT37/1	ABBBC
Tobacco	4013-5	D
Tobacco	4013	E

Fig. 5. Summary of T-DNA Structures in crown gall tumors.
On the left is a list of crown gall tumors incited by wildtype *Agrobacterium tumefaciens* octopine strains with the exception of the three lines preceeded by an asterisk (*) which were incited by wildtype nopaline strains. The letter or letters under the column headed 'T-DNA' indicate the structure or structures of the T-DNA in that line which were established by Southern blot analysis. We did not include data from tumors incited by insertion mutants or the data concerning the right T-DNA of the octopine Ti plasmid. The letters indicate whether the data is consistent with the T-DNA ending at the termini sequences (A), not reaching the left terminus sequence (B), not reaching the right terminus sequence (C), not reaching either terminus sequence (D) or extending past the left terminus sequence (E). On the right are schematic illustrations of each type of T-DNA structure. They are not drawn to scale. The structures are derived from the following references: 45 (A6S/2, B6806/E9, 15955/1, A277/5); 15 (Tu-B6S3, WB-B6S3, SR1-B6S3, WB-A6, A-Ach5, P-Ach5); 35 (4013-1; 4013-2, 4001, 4229, 4013-5, 4013); 33 (Vertus ACH5, Vertus B6 oct+, Vertus B6 oct-); 46 (PSCG15955); 31 (W38C58-1, BT37); 50 (LBA4013-4); 2 (Bla4); 30 (1595501); 22 (FT37/1).

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plasmid. The letters
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.-B6S3, WB-A6, A-
); 46 (PSCG13955):

sitive indicator of transfer near the left terminus sequence. Published DNA sequence analysis of the extent of T-DNA transfer from wild type nopaline Ti plasmids showed that one T-DNA ended in the left terminus sequence and included this EcoRI site but three others ended about 20 to 30 basepairs to the right of this EcoRI site (51, 53). Our analysis did not include restriction enzyme sites closer than about 1.5 kb from the right terminus sequence. However, the roots did synthesize nopaline and Shaw *et al.* (41) have shown that the nopaline synthase promotor requires sequences including those less than 350 basepairs from the right terminus sequence. Also, all four of the nopaline T-DNAs, which were characterized by DNA sequence analysis, ended within one base of the right terminus sequence (53).

The analysis of DNA from transformed roots indicates that the termini sequences of *A. tumefaciens* can function in *A. rhizogenes*. The termini sequences and other interacting factors appear to be functionally interchangable between octopine and nopaline wide-host-range Ti plasmids, octopine narrow-host-range Ti plasmids, and Ri plasmids (3, 17, 24, 25). Recent DNA sequence analysis confirms that Ri and Ti plasmids have similar termini sequences (42).

At least 3 of the 12 root cultures that we examined contain a minimum of one 'aberrant' copy of the vector T-DNA. This frequency can be compared to the frequency of aberrant copies from wild type Ti plasmids which we have summarized from the literature in Fig. 5. The structure of T-DNA copies (based on Southern blot analysis) in 26 tumors is indicated by letters. Data consistent with a 'perfect' transfer (A) is found in 16 cases but in 9 cases, at least one copy of the T-DNA does not reach the left terminus sequence (B) or does not reach the right terminus (C) or does not reach either terminus (D). In one case the T-DNA stretched past the left terminus sequence (E). Thus, the frequency of faithful transfer from our vectors is similar to the frequency with wild type Ti plasmids.

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EXHIBIT C

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Techniques

Induction of Hairy Roots on Cultivated Soybean Genotypes and Their Use to Propagate the Soybean Cyst Nematode

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ABSTRACT

Savka, M. A., Ravillion, B., Noel, G. R., and Farrand, S. K. 1990. Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode. Phytopathology 80:503-508.

Ten soybean (*Glycine max*) genotypes were evaluated for hairy root induction by four strains of *Agrobacterium rhizogenes*. Influence of inoculei in site was assessed by infecting hypocotyls and cotyledons on germinated seedlings. The presence of opines in extracts of cultured roots was used to score transformed roots. A cucumopine strain, K599, induced hairy roots on 37% of the cotyledons infected on the 10 genotypes tested. Transformed root development after infection of cotyledons with the mannopine strain 8196 occurred at a frequency of 3% on four genotypes. Agropine strains 1855 and A4 induced hairy roots on 1% of cotyledons of different genotypes. No opine-positive transformed roots were induced from hypocotyl inoculations with any *A. rhizogenes* strain-soybean genotype combination tested. However, adventitious roots containing no detectable opines developed from hypocotyl inoculations both at the wound site and at a region directly below the cotyledons. Transf rmed roots differentiated from globular callus at the wound site on cotyled ns infected with virulent *A. rhizogenes*. Opine-containing hairy roots were established permanently in tissue culture and exhibited typical hairy ro t morphologies and growth parameters. Infection of soybean cultivar Williams 82 hairy root cultures with second-stage juveniles or cysts f the soybean cyst nematode, *Heterodera glycines* race 3, led t the appearance of mature cysts about 3 wk later. The nematode was propagated by excising an infected root and transferring it to a fresh root culture.

Agrobacterium rhizogenes, the causal agent of hairy root disease, induces the proliferation of neoplastic, transformed roots (1,35,37). During infection, the T-region, a segment of the root-inducing (Ri) plasmid in *A. rhizogenes*, is transferred and stably integrated into the plant genome (5). Upon expression of this integrated T-DNA, transformed roots rapidly proliferate and synthesize certain low molecular weight carbon compounds called opines (25). F ur pine-type Ri plasmids have been identified. Agropine-, mannopine-, cucum pine- and mikimopine-type Ri plasmids harbored in strains of *A. rhizogenes* induce transformed roots which synthesize the strain-specific opines (7,11,13,26).

Recently, hairy r ot cultures have been used to cultivate obligate root parasites. *Plasmodiophora brassicae* Woronin and *Polymyxa betae* Keskin, both bligate r ot-inhabiting fungi, can be propagated on transformed root cultures f sugar beet (19). Infections with vesicular-arbuscular mycorrhizal fungi, *Glomus mosseae* Gerdemann & Trappe and *Gigaspora margarita* Beker & Hall, have been obtained on hairy root cultures of *Convolvulus septum* L. (20). In addition, the root-knot nematode, *Meloidogyne javanica*, has been propagated on transformed root cultures f potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.) (32). Such cultures are being used f r routine maintenance of the nematode and to study the parasitism f *M. javanica* by *Pasteuria penetrans* (Thorne) Sayre & Starr (33).

Soybean (*Glycine max* (L.) Merr.) is grown widely in the United States as a source of oil and high-protein meal. Annually, the soybean crop is valued at an estimated 11 billion dollars. *Heterodera glycines* Ichinohe, the s ybean cyst nematode, occurs in Canada, the Peoples' Republic of China, Colombia, Ind nesia, Japan, Korea, the Soviet Union, and throughout the s ybean producti n areas of the United States (29). This obligate ro t parasite is a maj r yield-limiting pest of soybean in the United States (12).

The soybean cyst nematode can be propagated gnotobiotically on normal soybean root explants (14). However, this technique requires the continual establishment of new explants because these organs have a determinant period of growth in culture. Soybean hairy roots, which should exhibit indeterminate growth in tissue culture, could provide an alternative to normal root explants for monoxenic propagation and study of obligate soybean root parasites such as the soybean cyst nematode.

The few reports in the literature suggest that *A. rhizogenes*-induced hairy roots are difficult to establish on soybean. Responses of 26 genotypes of *G. max* to induction of hairy roots by *Agrobacterium* strain A136 harboring pRiA4b have been reported (23). Seven of the genotypes produced roots at the infected wound sites, another eight produced only small galls, and the remaining 11 did not respond to inoculations with this bacterial strain. However, attempts to culture these roots were unsuccessful. In addition, primary roots were not characterized with respect to nicotine content or other hairy root markers (23). Recently, Reich and co-workers (28) induced hairy roots on *G. canescens*, a wild *Glycine* spp. Permanent cultures could be established and the transformed roots were regenerable. However, hairy root cultures of the domesticated genotypes of *G. max* have not yet been reported.

This paper describes 1) an investigation into genotype, pathogen, and infection parameters necessary to induce hairy roots on *G. max*, 2) the establishment and characteristics of soybean hairy root cultures, and 3) the use of these cultures for the axenic propagation of the soybean cyst nematode.

MATERIALS AND METHODS

Soybean genotypes. The 10 genotypes of *Glycine max* used in this study were acquired from R. L. Bernard, curator, USDA Northern Soybean Germplasm Collection, University of Illinois at Urbana-Champaign, Urbana. Soybean seeds were surface sterilized by soaking in 2.1% sodium hypochlorite for 20 min followed by two 5-min washes in sterile distilled water. Seeds then were plated onto sucrose water agar (5.0% sucrose in 0.8% agar) medium (SWA) to allow germination and to select for sterile seeds. Germinating seeds were transferred to 25- × 150-mm test tubes containing 10 ml of SWA.

Bacteria. Four strains of *A. rhizogenes* were evaluated for their ability to induce transformed roots on 10 soybean genotypes. Two agropine-type strains, A4 and 1855, and one mannopine strain, 8196, were from our collection. The cucumopine strain, K599, was obtained from Allen Kerr, Waite Institute, Glen Osmond, 5064—South Australia. Nonpathogenic strain NT-1 is *A. tumefaciens* strain C58 cured of its Ti plasmid (34). Bacterial strains were grown in yeast extract-mannitol liquid medium (27) with aeration at 28 C.

Plant inoculations. Soybean seedlings were inoculated after the emergence vegetative stage (10). The onset of vegetative stage in the 10 selected soybean seedling genotypes varied between 6 and 15 days after plating seed on SWA. Inoculations were performed with a scalpel previously dipped into an overnight culture of the strain of *Agrobacterium* being tested. Cotyledons were inoculated by cutting the abaxial face several times to form a checked wound site. Hypocotyl segments were inoculated by making 2.0-cm-long longitudinal cuts. Twenty seedlings of each genotype were inoculated at each site for each bacterial strain tested. Inoculated seedlings were returned to 25- × 150-mm test tubes and incubated in growth chambers under cool-white fluorescent lighting for a 16-hr photoperiod at 25 C.

Establishment of root cultures. Cotyledons and hypocotyls with root primordia were transferred to 25 ml of liquid MonMor medium in 25- × 100-mm culture plates. MonMor medium consisted of Monnier's salts (17) containing Morel's vitamins (18), 86 mg L^{-1} of ferric-sodium salt EDTA according to Murashige and Skoog medium (21) and 20.0 g L^{-1} of sucrose. The pH was adjusted to 5.8 before autoclaving for 20 min at 118 C and 1.0 g cm^{-3}. After autoclaving, the medium was cooled to approximately 45 C and carbenicillin at 500 mg L^{-1} was added to inhibit

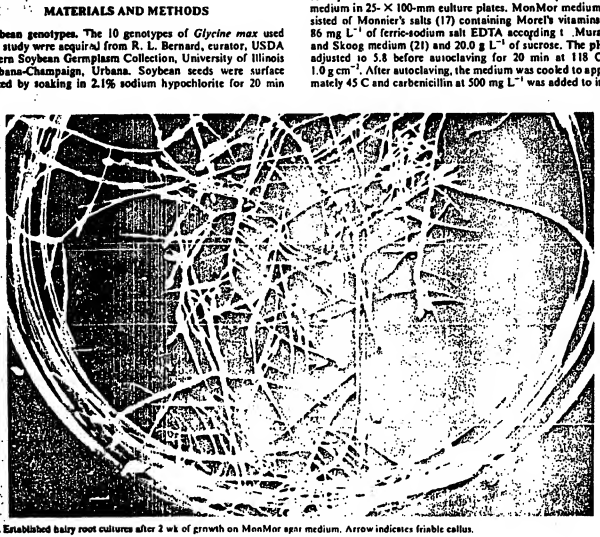

Fig. 1. Established hairy root cultures after 2 wk of growth on MonMor agar medium. Arrow indicates friable callus.

bacterial growth. For propagation of the soybean cyst nematode, approximately 2.0 g of verified transformed roots were subcultured from MonMor liquid medium to Lauritis medium (14,15) containing 16.0 g L^{-1} of Difco Bacto agar in 150- × 25-mm culture plates.

Opine analysis. For the detection of the mannityl opines, approximately 0.3 g of root tissue was macerated in 100 µl of 50% ethanol containing 10 µl of the electrophoresis running buffer (formic acid/acetic acid/water, 3:6:91, v/v/v, pH = 1.9). For detection of cucumopine, root tissue was macerated in distilled water. In each case supernatants were recovered following centrifugation. Twenty microliters of supernatant extract was spotted onto Whatman 3 MM paper. The spots were allowed to dry, and the papers were wetted with the running buffer and subjected to high voltage paper electrophoresis (HVPE) at 4,000 V for 12–15 min. The electrophoretograms were dried in a stream of warm air until no odor of acetic acid could be detected.

TABLE 1. Frequency of hairy root induction on cotyledons of genotypes of *Glycine max* inoculated with one of four strains of *Agrobacterium rhizogenes*

Genotype	Opine positive roots / total roots[1] *A. rhizogenes* strain			
	K599	8196	1855	A4
Cartter	13/13[2]	0/0	1/2	0/3
Fayette	10/10	1/3	0/2	0/0
Franklin	1/1	0/0	0/0	0/0
Kent	10/10	1/3	0/0	0/0
Lee	3/3	0/0	0/0	0/0
Mandarin	17/17	1/2	0/0	1/2
Maple Arrow	15/15	2/4	1/3	1/6
Peking	1/1	0/0	0/0	0/0
Pickett	1/1	0/0	0/0	0/0
Williams 82	3/3	0/2	0/0	1/3
Total	74/74/200[3]	5/14/200	2/7/200	3/14/200

[1] In each case 20 cotyledons were inoculated with each strain of *A. rhizogenes*.
[2] Number of cotyledons yielding opine-positive roots/number of cotyledons producing roots at the wound site.
[3] Total number of cotyledons inoculated by each strain.

Mannityl opines were visualized with the alkaline silver nitrate reagents of Trevelyan and co-workers (31). Electrophoretograms were dipped in silver nitrate solution (4 g of silver nitrate in 20 ml water diluted to 1 L with acetone) and dried thoroughly. The spots were developed by dipping in ethanolic NaOH (2% NaOH in 90% ethanol). The papers were subsequently dipped in Kodak fixer and rinsed with distilled water for 15 min (6).

Cucumopine and its acid-degradation product were visualized with the Pauly reagent by spraying the dry electrophoretograms lightly with a solution containing equal parts of sulfanylic acid (1.0% in 1 N HCl) and sodium nitrite (5.0% in water). Papers were allowed to dry and then sprayed with aqueous 15% sodium carbonate (8,24). Cucumopine and its acid-degradation product appear as reddish and bluish spots, respectively, as the paper is sprayed with sodium carbonate.

Spots were identified as opines by comparing their electrophoretic mobilities and staining properties with those of authentic standards. Mannopine, mannopinic acid, agropine, and agropinic acid were synthesized by Yves Dessaux in our laboratory. Cucumopine was synthesized from L-histidine and α-ketoglutaric acid (7) by Paul Hanselmann, also in our laboratory. Extracts prepared from normal leaf or root tissues or from authentic hairy roots of *Nicotiana tabacum* L. 'Xanthi NG' were included on electrophoretograms as negative and positive controls, respectively.

Propagation of *Heterodera glycines*. Soybean cultivar Williams 82 transformed root cultures, freshly transferred to plates containing Lauritis medium (14), were inoculated with six to eight gravid females of *H. glycines* race 3 from gnotobiotic culture (15). Alternatively, second-stage juveniles (J2) from pot cultures were collected and surface sterilized by soaking in a solution containing 100 mg L^{-1} of $HgCl_2$ and 1,000 mg L^{-1} of sterile streptomycin sulfate. Nematodes were washed twice with sterile distilled water by centrifugation (16). Between 50 and 100 J2 were added to the subcultured transformed root cultures grown on Lauritis medium.

RESULTS

Differentiation of roots at inoculated sites. After approximately 10 days, globular callus tissue appeared at some of the wound sites of cotyledons inoculated with strains of *A. rhizogenes*. Extensive splitting of hypocotyls with no callus formation occurred

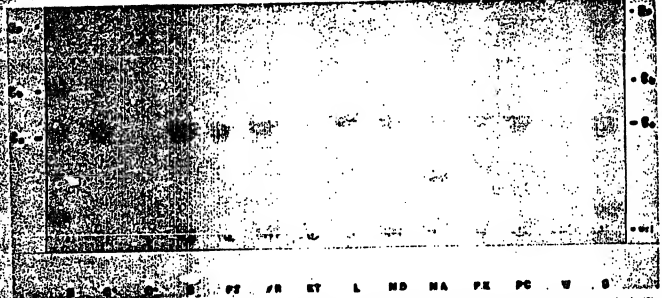

Fig. 2. Electrophoretic analysis of extracts from transformed roots of soybean incited by *Agrobacterium rhizogenes* strain K599. Standards (Sav): cucumopine (Ca), acid-degradative product of cucumopine (Cb), and histidine (Hp). (C+), Extract of tobacco hairy roots induced with cucumopine strain K599. (C-), Extract from normal tobacco roots. Other lanes contain root extracts from roots induced on genotypes: Cartter (C), Fayette (FT), Franklin (FR), Kent (KT), Lee (L), Mandarin (MD), Maple Arrow (MA), Peking (PK), Pickett (PC), and Williams 82 (W).

on inoculated hypocotyls with all bacterial strains tested. Fifteen to 25 days after inoculation on cotyledons with strains of *A. rhizogenes*, root primordia differentiated from globular callus tissue. Hypocotyls inoculated with virulent strains of *A. rhizogenes* or the nonpathogenic strain NT-1 gave rise to roots at the inoculation site and at a region about 0.5 cm below the cotyledons. Roots that developed from hypocotyls did not contain detectable opines in their cell extracts (data not shown).

When root primordia had elongated to approximately 2.0 cm, the entire hypocotyl or cotyledon was dissected from the seedling and transferred to liquid MonMor medium containing carbenicillin. Approximately 10% of the roots failed to grow in liquid MonMor medium after excision from the seedling. After 1 wk, clonal lines were established by subculturing single roots. While some subcultured roots failed to elongate, most of the roots showed growth rates of approximately 0.5 cm per 24 hr. When transferred to solid medium, many of the roots formed a small amount of friable callus at root tips (Fig. 1).

Roots containing opines were scored as being transformed (see below). Hairy root cultures were established by subculturing 4-cm segments of root meristem to 25 ml of liquid or solid MonMor medium. Hairy root cultures could be routinely maintained on solid MonMor medium by subculturing at 3-wk intervals. Hairy root cultures agitated at 60 rpm in liquid MonMor medium grew rapidly and subculturing was necessary every 10 days.

Efficiency of different strains of *A. rhizogenes*. Strain K599 was the most efficient at inciting hairy roots on cotyledons of the 10 soybean genotypes tested. This strain induced transformed roots on 75–85% of the infected cotyledons, depending on genotype (Table 1). Cucumopine, the indicator opine associated with tissues transformed by strain K599, was present in extracts from all roots tested (Fig. 2).

Root formation following inoculation with agropine strains 1855 and A4 occurred at frequencies of 3 and 7%, respectively (Table 1). However, the absence of opines in extracts indicated that most of these roots were not truly transformed (Table 1 and Fig. 3). Mannopine strain 8196 induced roots at a frequency of 7%, and only 35% of these were found to contain mannopine and mannopinic acid (Table 1 and Fig. 3).

Soybean genotypes. Efficiency of transformed root induction on cotyledons by strain K599 varied among the 10 soybean genotypes evaluated. Two genotypes, Mandarin and Maple Arrow, were quite responsive, yielding hairy roots in 75–85% of the infected cotyledons. Other genotypes, such as Franklin, Peking and Pickett, were relatively insensitive, showing infection rates of less than 10%.

Propagation of *glycines* race 3. Twenty to 25 days after inoculation (DAI) with gravid females and 16–20 DAI with J2, imbedded and emerging females were observed on Williams 82 hairy roots induced by strain K599 (Fig. 4A). Approximately 4–6 days after cyst emergence, first molting was observed followed by egg hatch and emergence of J2 (Fig 4B). Second-stage juveniles were observed migrating throughout the culture (Fig. 4C) and mature second-generation females were observed approximately 6 wk after inoculation (Fig. 4D). The nematode could be serially propagated by transferring infected hairy root segments to a fresh hairy root culture (data not shown).

DISCUSSION

The three variables tested, host genotype, strain of *A. rhizogenes*, and site of inoculation all proved important in the successful induction of hairy roots on soybeans. In general, cotyledon inoculations were more effective than stem or hypocotyl infections. This contrasts with results reported by Owens and Cress (23) who showed that stem inoculations were more effective than cotyledon infections. However, they did not characterize roots appearing at inoculation sites. Our observations that hypocotyl wound sites give rise to normal adventitious roots raises the question as to whether the roots appearing at their infection sites were truly transformed. In fact, our observations suggest that the genotypes of *G. max* tested have a propensity to form adventitious roots when inoculated with strains of *Agrobacterium*. This response depends on inoculation of *Agrobacterium* but does not require an Ri plasmid. Hypocotyl infections with strain NT-1 regularly gave rise to root proliferation at the wound sites and at a nonwounded collar region just below the cotyledons. Such roots from plants infected by *A. rhizogenes* and from plants infected with strain NT-1 contained no detectable opines. A few adventitious roots also developed from inoculated cotyledons. However, in such infections the nontransformed roots generally arose at the junction between the cotyledon and its petiole, distant from the actual wound sites. The roots forming at the wound site usually were transformed as judged by the presence of the marker opines.

Hairy root induction depended on the strain of *A. rhizogenes*. Strain K599 was by far the most effective in inducing hairy roots, with all soybean genotypes tested being sensitive to infection by this strain. The one mannopine-type and the two agropine-type strains of *A. rhizogenes* tested were much less effective at inducing hairy roots on soybeans (Table 1). These results are consistent with those of Byrne and co-workers (2) who failed to observe

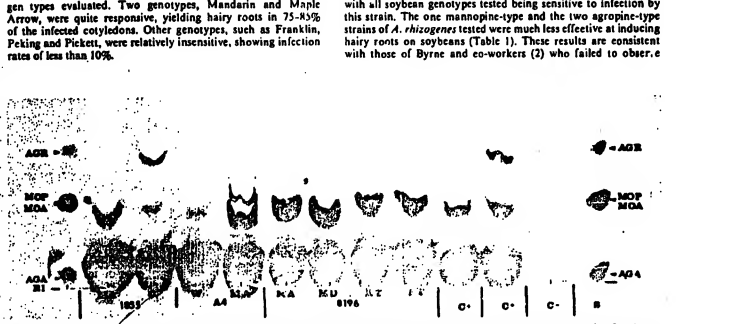

Fig. 3. Electrophoretic analysis of extracts from transformed roots incited by mannopine and agropine-type *Agrobacterium rhizogenes* strains. Standards (S) are: agropine (AGR), mannopine (MOP), mannopinic acid (MOA), and agropinic acid (AGA). Mannopine and mannopinic acid comigrate under these electrophoretic conditions. Other lanes contain root extracts from: Maple Arrow (MA) and Cartier (C) induced by strain 1855; Williams 82 (W) and Maple Arrow (MA) induced by strain A4; Maple Arrow (MA), Mandarin (MD), Kent (KT) and Fayette (FT) induced by strain 8196. (C+), Extracts from tobacco hairy roots incited by strains 8196 and 1855. (C−), Extracts from normal tobacco roots.

tiny hairy r ot induction on 17 genotyp s of [redacted] er by a strain of *Agrobatterium* containing pRi8196. Nor did strain 8196 induce hairy roots on *G. soja* r *G. canescens*. This is consistent with our bservati n that strain 8196 shows p or hairy r ot induction on the genotypes f *G. max* we tested (Table 1). However, our results contrast with experiments reported by Pech et al (28) on transformati n f ther *Glycine* spp. They bserved that, ith ugh frequencies varied, a strain harb ring the agropine-type Ri plasmid, pRi1855, was highly effective in transforming several accessions f *G. canescens, G. clandestina*, and *G. argyrea*. They also found hyp cotyls to be more responsive than cotyledons. These differences may be due to dissimilarities in host plant species, chromosomal backgrounds of the bacteria, cultural conditions, or a combination of the three factors.

Hairy root formation also depended on the host plant genotype. Based on frequencies at which opine-positive roots arose, the 10 soybean genotypes tested could be divided into two groups. Genotypes Cartter, Fayette, Kent, Mandarin, and Maple Arrow were judged t be sensitive, showing frequencies of hairy root formation by strain K599 ranging from 50 to 85%. The remaining gen types were relatively insensitive with transformation frequencies by this strain below 20%. Although the numbers are low, the few productive infections with the agropine- and mannopine-type strains of *A. rhizogenes* occurred most frequently on those gen types susceptible to infection by strain K599 (Table 1).

Roots at wound sites were judged as transformed if opines were detected in cell-free extracts. Such opine-positive roots generally exhibited other [redacted] notypes associated with true hairy r ots including fast growth in culture, loss f geotropism, and lateral root branching (Fig. 1; 22,30). N morphol gical differences were noted among opine-positive roots of various *G. max* genotypes. When established in tissue culture, opine-positive hairy roots retained their transformed phenotypes. Furtherm re, axenic root cultures c uld be maintained f r at least 1 yr by transferring root tip cuttings fr m older cultures t fresh medium.

While id ntification based on opine content is sound for analysis of roots induced by the cucumopine and mannopine strains, it may underestimate the frequency f transformation by agr pine strains. This is because, unlike cucumopine and mann pine strains, the opine biosynthetic genes in the agropine-type Ri plasmids are encoded on a T-DNA segment separate from that which encodes the *onc* genes (9,36). Thus, it is possible that s me of the roots resulting from infection by the agropine strains were transformed but contained only the oncogenic T-DNA segment (3,4). However, the two agropine strains tested were inefficient at inducing either adventitious or transformed roots at w und sites (Table 1).

Hairy root cultures of Williams 82 inoculated with *H. glycines* race 3 produced mature cy ts approximately 21 days after nematode inoculation (Fig. 4B). Root cultures could be infected with gravid females or with J2, although inoculation with the f rm r was simpler and appeared to be more efficient. The time required for development of mature cysts was similar to that reported for *H. glycines* on axenic explant cultures of normal soybean

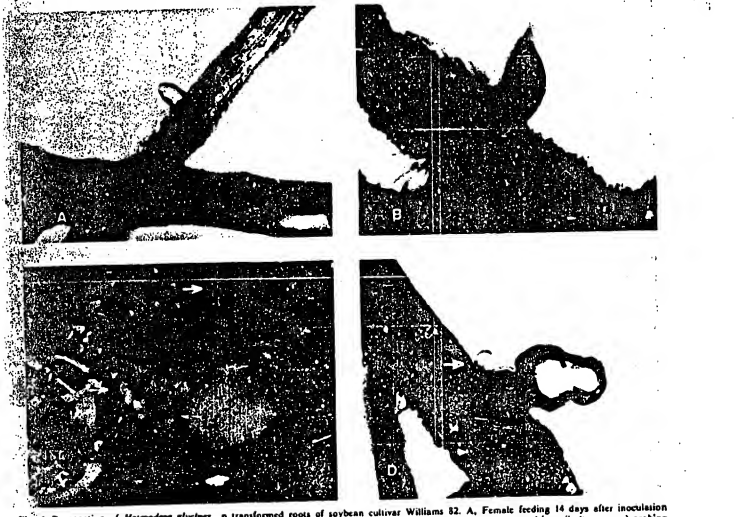

Fig. 4. Propagati n f *Heterodera glycines* n transformed roots of soybean cultivar Williams 82. A, Female feeding 14 days after inoculation with second-stage juveniles. B, Female 21 days after inoculation. C, Emergence of second-stage juvenile from cyst and juvenile (top arrow) probing root surface. D, Migrating juvenile (arr w).

roots (14,15). After an additional week, second-generation cysts were observed, indicating that the nematode could complete its entire life cycle in transformed root cultures (Fig. 4A).

Hairy root cultures may provide some advantages over normal root explants for monoxenic culture of *H. glycines*. First, transformed roots grow indefinitely in tissue culture by virtue of the need to periodically reestablish new root explants from germinating seedlings. Furthermore, because the transformed roots are clonal in origin, established hairy root cultures should assure a uniformity in genetic background. Second, hairy root cultures may enhance reproductive capacity of the nematode. Such was the case for the propagation of *M. javanica* on cultured tomato hairy roots (33). This increase in reproduction was ascribed to the large numbers of lateral roots produced by the transformed tissues (33). Root branching also is characteristic of soybean hairy root cultures (Fig. 1). Third, since the *A. rhizogenes* system provides a way to insert new genes into differentiated tissues, novel genes conferring nematode resistance or the biosynthesis of potential control compounds could be engineered into the soybean genome and directly tested for their efficacy in conferring resistance to *H. glycines*. Finally, a simple method to axenically cultivate the soybean cyst nematode could be of considerable value in the study of the molecular biology and genetics of *H. glycines*.

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X. RELATED PROCEEDINGS APPENDIX

There are no related proceedings.